CHARACTERIZATION OF GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE FROM GROUP A STREPTOCOCCI AND ANALYSIS OF ITS ROLE AS A PLASMIN RECEPTOR

By

SCOTT BUDD WINRAM

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1995

This work is dedicated to my dog, Booger, without whose lessons in blind faith this work would not have been possible.

ACKNOWLEDGMENTS

I would like to thank Dr. Richard Lottenberg for his guidance and friendship during my thesis work. I would also like to thank my outside examiner Dr. Donald Leblanc and the members of my committee for their help in guiding this project, especially Dr. Paul Gulig who contributed significantly to my graduate education. Most of all I would like to acknowledge my parents, Geraldine Holt, and Kathleen O'Donnel for their unwavering love and support.

TABLE OF CONTENTS

Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

CHARACTERIZATION OF GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE FROM GROUP A STREPTOCOCCI AND ANALYSIS OF ITS ROLE AS A PLASMIN RECEPTOR

By

Scott Budd Winram

August 1995

Chairman: Richard Lottenberg, M.D.
Major Department: Molecular Genetics and Microbiology

Group A streptococcus is a human pathogen which has the capacity to cause highly invasive soft tissue infections. Streptococci can interact with the plasminogen system by secreting the plasminogen activator streptokinase and subsequently capturing the serine protease plasmin on the cell surface. To assess the contribution of this surface proteolytic activity to the invasive potential of the organism, receptor molecules must be identified. Plr, a candidate plasmin receptor protein from group A strain 64/14, has previously been isolated and the gene encoding this protein, *plr*, cloned in our laboratory. In this work, Plr, has been identified as a functional glyceraldehyde-3-phosphate dehydrogenase and characterized both biochemically

and genetically. Plr was the only GAPDH identified in strain 64/14 and *plr* appears to be an essential gene. Analysis of expressed products of mutated *plr* revealed that a lysine in the C-terminal position was necessary for wild-type levels of plasmin binding. Additional residues of Plr also appear to participate in the binding interaction with plasmin. Plasmin-binding deficient Plr molecules with alterations of the C-terminal lysine retained GAPDH enzymatic activity. The genes harboring these mutations have been introduced successfully into group A strain 64/14 at the *plr* locus. Streptococcal strains expressing the mutant Plr molecules and wild-type streptococci demonstrated equivalent plasmin binding activity. Additional investigations indicated that the majority of plasmin receptors on strain 64/14 are proteins and that C-terminal lysines are required for at least some of the binding activity. Therefore, strain 64/14 expresses plasmin binding structures other than Plr. The role of plasmin receptors in streptococcal pathogenesis remains to be determined.

INTRODUCTION

Group A Streptococci

Group A streptococci are Gram-positive bacteria capable of causing a variety of diseases in humans ranging from acute pharyngitis to potentially lethal invasive soft tissue infections and a toxic shock-like syndrome (Stevens, 1992). Rheumatic fever and glomerulonephritis are two serious post-infectious sequelae of group A streptococcal infections (Bisno, 1991). Group A streptococci possess many surface and secreted factors that may contribute to virulence, but few have been definitively identified as participating in the in vivo pathogenesis of streptococci.

Surface structures that have been identified include the hyaluronic acid capsule, streptolysins O and S, M-protein and M-related proteins such as the IgG binding proteins, fibronectin binding proteins such as Protein F, collagen binding proteins, C5a peptidase, and plasmin(ogen) receptor(s). The capsule mimics host cell surfaces and thereby aids the bacteria in avoiding opsonization and phagocytosis. The importance of the capsule in the virulence of group A streptococci was demonstrated when transposon

mutagenesis of the region encoding genes required for capsule synthesis resulted in a 100-fold decrease of virulence in mice (Wessels et al., 1991). In addition to binding several host proteins including fibrinogen, the surface M-protein has also been shown to inhibit opsonization and phagocytosis of streptococci in vitro. Over 80 antigenically distinct types of M-protein have been identified to date. Opsonic antibodies are usually made against M-protein at a variable region in the exposed NH_2-terminus of the protein. Because of this, protective antibodies against one serotype of group A streptococci still leaves the host susceptible to infection by the remaining serotypes (reviewed by Fischetti, 1989). The fibronectin binding protein, Protein F, has been identified as an adhesin of group A streptococci (Hanski and Caparon 1992). The cloned gene product expressed in *E. coli* increased adherence of the bacteria to fibronectin. Lipoteichoic acid on the bacterial surface has also been reported to bind fibronectin (Simpson and Beachey, 1983). Recently it has been recognized that certain environmental stimuli can modulate expression of streptococcal surface proteins. The genes encoding M- and M-like proteins and the C5a peptidase reside together in an operon which is regulated in response to environmental CO_2 tension (Okada et al. 1993). By growing streptococci strains under high or low CO_2 concentrations to increase or repress M-protein expression, preferential binding was demonstrated to skin keratinocytes or to

Langerhans cells. Investigations using isogenic strains indicated that Protein F was the Langerhan cell adhesin and suggested that the character of the bacterial surface could be altered during various stages of invasive infections (Okada et al., 1994).

Many group A streptococcal strains also secrete several toxins such as SpeA, SpeB, and SpeC. SpeA and SpeC have been shown to have toxic effects in animals and act as T-cell mitogens in vitro (Lee and Schlievert, 1989; Drake and Kotzin, 1992). Epidemiologic studies have indicated that the strains causing the toxic shock-like syndrome often express these toxins (Hauser et al., 1991). The active form of SpeB has been reported to be a cysteine protease and is able to cleave the IL-1 cytokine precursor as well as the urokinase plasminogen activator receptor (Kapur et al., 1993; Wolf et al., 1994). The role of these secreted factors and their regulation in the pathogenesis of group A streptococci remains to be elucidated.

Group A streptococci have the potential to interact directly with the human plasminogen system. These bacteria secrete streptokinase which is a potent activator of plasminogen (Castellino, 1979). Plasmin can degrade a wide range of protein substrates including fibrin and other protein that comprise tissue planes, and group A streptococci can bind enzymatically active plasmin on the cell surface (Lottenberg et al., 1987). These interactions of group A streptococci with the plasminogen system may contribute to

the ability of the bacteria to traverse the extracellular matrix during invasive infections but the contribution of these interactions has not been defined.

The Plasminogen System

The zymogen plasminogen contains 791 amino acids in its mature form. Plasminogen is found in blood, saliva, and in extracellular spaces. Plasminogen is converted into the active serine protease plasmin by either host or bacterial plasminogen activators via cleavage of the peptide bond between arginine^{561} and valine^{562} (reviewed by Vassalli et al., 1991). The human plasminogen activators, tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA), possess proteolytic activity. In contrast, the prokaryotic plasminogen activators streptokinase and staphylokinase, secreted by certain strains of streptococci and staphylococci, respectively, have been reported to initially form a one to one stochiometric complex with plasminogen and this complex then converts other molecules of plasminogen to plasmin (Castellino, 1979). The plasminogen molecule consists of a heavy chain and a light chain linked together by three disulfide bonds. The light chain contains an enzymatic active site with an amino acid sequence of a serine protease. The heavy chain contains five well characterized loops referred to as kringles, which serve as

lysine binding sites (LBS) for other proteins. There are two classes of lysine binding sites. Kringle 1 has a high affinity binding site for lysine whereas the low affinity sites are localized to kringles 2 through 5. The native NH_2-terminal amino acid of secreted plasminogen is glutamic acid (glu-plasminogen). Plasmin will cleave the peptide bond between $lysine^{77}$ and $lysine^{78}$ of glu-plasmin(ogen) to form lys-plasminogen or lys-plasmin (reviewed by Castillino, 1995). The conversion of glu-plasminogen to lys-plasminogen results in a dramatic conformational change. The interaction of lysine or epsilon-amino-caproic acid (EACA) with glu-plasminogen will also produce this conformational change (Violand et al., 1975). This "lys" conformation has been reported to be required for high affinity binding interactions through a C-terminal lysine residue of some ligands such as fibrin fragments and alpha-2-antiplasmin (Christensen, 1988; Sasaki et al., 1986).

Plasmin has trypsin-like specificity, with the ability to hydrolyze peptide bonds after lysine and arginine residues. This protease has been characterized most thoroughly for its role in the dissolution of fibrin clots, but has also been shown to degrade a variety of proteins, including laminin and fibronectin which comprise the extracellular matrix. Plasmin also activates latent metalloproteases such as collagenase which further amplifies plasmin's degradative effects (Vassalli et al., 1991). The inhibitor alpha-2-antiplasmin binds plasmin via the high

affinity LBS on kringle I and the active site, and is the primary physiological regulator of plasmin (Aoki, 1995).

Plasmin(ogen) Receptors

A wide range of eukaryotic cells express surface receptors for plasmin(ogen) and plasminogen activators. Some of the eukaryotic cells which have been identified as binding plasmin(ogen) include platelets, monocytes, lymphocytes, microglial neurons, human keratinocytes, colonic and breast carcinoma cell lines, and monocytoid cell lines (Miles and Plow, 1988; Nakajima et al., 1994; Burge et al., 1992; Burtin et al., 1988; Miles et al., 1991). Several specific receptor molecules identified on these cell types are GPIIb/IIIa on platelets (Miles et al., 1986), the glycolytic enzyme alpha-enolase on U937 monocytoid cells (Miles et al. 1991) and on the plasma membrane of rat microglial cells (Nakajima et al., 1994), and recently a protein homologous to alpha-enolase was identified on the colonic cell line SW1116 (Lopez-Alemany et al., 1994). Surface bound plasmin remains proteolytically active and is protected from inhibition by alpha-2-antiplasmin. Urokinase and tPA have also been shown to bind to cells which enhances plasminogen activation and cell-bound plasmin activity. Receptor molecules have been identified for urokinase, the urokinase plasminogen-activator receptor (uPAR), and for tPA, annexin II (Vassalli et al., 1985;

Hajjar et al., 1994). The cell-bound plasmin and plasminogen activators are hypothesized to play a role in the migration of cells through the extracellular matrix for physiological processes such as embryogenesis as well as in pathological events including the inflammatory response of the host and metastatic spread of tumor cells.

In addition to the eukaryotic plasmin(ogen) receptors, there is a wide range of both Gram-positive and Gram-negative bacteria that have been reported to bind plasmin(ogen) including group A, C, and G streptococci, *Staphylococcus aureus*, *Proteus mirabilis*, *Haemophilis influenza*, *Pseudomonas aeruginosa*, *Neisseria meningitidis* and *Neisseria gonorrhea*, *Escherichia coli*, and *Borrelia burgdorferi* (Ullberg et al., 1989; Ullberg et al., 1990; Ullberg 1992; Kuusela et al., 1990; Fuchs et al., 1994). Similar to the findings for eukaryotic cells, the surface-bound plasmin is not regulated by alpha-2-antiplasmin. In order to determine the role of bacteria-associated plasmin in the pathogenesis of infections caused by these organisms, the plasmin(ogen) binding components of the cell surface must first be identified. Several of the prokaryotic surface receptors for plasmin(ogen) which have been reported thus far have been the flagella and fimbriae of *E. coli* (Lahteenmaki et al., 1993; Parkkinen and Korhonen 1989), the OspA protein of *B. burgdorferi* (Fuchs et al., 1994), and certain M-related proteins of group A, C and G streptococci (Berge and Sjobring, 1993; Nasar et al., 1994).

Specific Background and Goals

Our laboratory was the first to demonstrate that group A streptococci can bind active plasmin with a high affinity on the bacterial surface (Lottenberg et al., 1987). We have also shown that group A streptococci grown in human plasma can activate plasminogen via streptokinase, and subsequently capture plasmin activity on the bacterial surface (Lottenberg et al., 1992b). Many group A streptococci, including strain 64/14, specifically bind plasmin or lys-plasminogen with a reduced affinity for native glu-plasminogen (Broder et al., 1989).

Analogous to the eukaryotic plasmin receptors, surface bound proteolytic activity can no longer be regulated by physiological plasmin inhibitors. This unregulated protease bound to the streptococcal surface may facilitate the movement of bacteria through normal host tissue barriers.

Recently, a candidate plasmin receptor protein was isolated from mutanolysin extracts of group A strain 64/14 by our laboratory (Broder et al., 1991). A gene encoding this protein, *plr*, has been cloned, sequenced, and expressed in *E. coli* (Lottenberg et al., 1992a). The predicted amino acid sequence of Plr exhibits extensive homology to the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

The goals of this study were to characterize Plr and to determine its contribution to the plasmin binding phenotype of group A streptococci. Although GAPDH molecules from a

wide variety of organisms had been extensively examined, GAPDH from pyogenic streptococci had not been characterized. Therefore this study addresses, on both a biochemical and a genetic level, the relationship between Plr isolated from mutanolysin extracts and the cytoplasmic GAPDH of group A streptococcal strain 64/14. Information generated from these studies was utilized in developing a strategy to generate isogenic mutant strains of group A streptococci. Genetic manipulation of recombinant *plr* in vitro yielded mutant Plr molecules that were deficient in plasmin binding activity relative to Plr, yet retained GAPDH enzymatic activity. These mutations were introduced into the group A strain 64/14 and the resulting strains were compared to wild-type bacteria for the ability to bind plasmin.

CHAPTER 1
THE RELATIONSHIP BETWEEN GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE AND PLR FROM GROUP A STREPTOCOCCI

Plr, a Mr ~41,000 protein, was previously identified as a candidate plasmin receptor from group A streptococcal strain 64/14 (Broder et al., 1989). The gene encoding this plasmin binding protein, *plr*, was cloned and the deduced amino acid sequence of Plr had extensive homology with the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Lottenberg et al., 1992a). The goal of this series of experiments was to characterize Plr from group A streptococci strain 64/14. Because of the homology of Plr to GAPDH, we hypothesized that Plr could be a functional GAPDH enzyme. Group A streptococci could contain multiple isozymes of GAPDH potentially serving different functions, and may harbor more than one gene encoding this protein(s). To definitively assess the role of Plr as a plasmin receptor, a genetic approach was required and therefore a more extensive characterization of Plr was necessary. I have isolated cytoplasmic GAPDH of streptococcal strain 64/14 and examined, on both structural and functional levels, its relatedness to strain 64/14 Plr obtained from mutanolysin extraction, and to recombinant Plr expressed in *E. coli*.

Glycolytic enzymes and the genes encoding them have not previously been examined in group A streptococci. Studies presented in this chapter demonstrated that Plr isolated from cell wall extracts by mutanolysin treatment was identical to GAPDH found in the cytoplasm of strain 64/14. Furthermore, this protein was encoded by a single open reading frame on the streptococcal chromosome and appears to be essential for viability. These studies have provided novel information about this glycolytic enzyme of group A streptococci and also a basis for the initiation of mutagenesis on *plr* studies presented in Chapters 2 and 3.

Materials and Methods

Bacterial strains and growth conditions. Group A streptococcal strain 64/14 is an M-untypable clinical isolate that has been previously mouse-passaged 14 times (Reis et al., 1984). Other Group A streptococcal strains used in this study are clinical isolates described previously (Wang et al., 1994). *E. coli* χ6060 [F' (*traD36 proAB lacI*^{q} *ΔlacZM15*)::Tn5 (Km^{r})/*araD139 Δ*(ara *leu) 7697 ΔlacX74 ΔphoA20 galE galK recA1 rpsE argE* (Am) *rpoB thi*] and *E. coli* χ2602 (LE392) [F- (*hsd R514* (r-, m+) *supE44, supF58, lacY1,* or *D*(*lacIZY*)*6, galK2, galT22, metB1, trpR55*] were used for transformation and gene expression.

Streptococci were grown as a standing culture to stationary phase at 37° C in Todd-Hewitt broth containing

0.3% wt/vol yeast extract (THY) or in a chemically defined medium (CDM) (Van DeRijn et al., 1980) containing 1% wt/vol glucose as the carbon source. In some experiments, streptococci grown to stationary phase were inoculated into CDM containing 0.5% wt/vol succinate and 0.5% vol/vol glycerol, 1% wt/vol acetate, or 1% wt/vol pyruvate in lieu of glucose. Standing cultures were incubated for several days at 37° C. *E. coli* χ6060 and *E. coli* χ2602 were grown as a shaking culture to stationary phase at 37° C. Antibiotics were added at concentrations of 10 µg/ml of kanamycin, 30 µg/ml chloramphenicol, and 34 µg/ml tetracycline where appropriate.

DNA manipulations and constructions of plasmids.

Plasmids were constructed using DNA techniques performed by standard methodology (Maniatis et al., 1989).

The plasmid pRL015 contains a 2.7 kb DNA fragment isolated from group A strain 64/14, which includes *plr* and its flanking chromosomal sequences (see insert of figure 1-5), ligated into the *Eco*RI restriction site of the low-copy cloning vector pYA2204 (Galan et al., 1988).

The plasmid pACYC184 is a medium copy *E. coli* cloning vector with both chloramphenicol and tetracycline resistance genes and the origin of replication from plasmid p15A (Chang and Cohen, 1978).

A 2.3 kb *Bam*HI-*Hind*III fragment from the 2.7 kb insert of pRL015 was subcloned into the *Bam*HI-*Hind*III restriction

sites of pACYC184 to generate pRL024. The plasmid pRL024 encodes chloramphenicol resistance (cm^r) and *plr* is transcribed in the opposite direction of the cm^r gene promoter.

The 2.3 kb *Bam*HI-*Hind*III insert of pRL024 was excised, blunt ended, and subcloned into *Pvu*II digested pACYC184 to generate pRL026. The putative promoter of *plr* transcribes in the opposite direction to the tet^r promoter of pRL026.

A 2.3 kb *Eco*RI fragment containing a Ω kanamycin resistant (Km^r) gene cassette was blunt ended and subcloned into the unique *Pvu*II site of pRL026 located within the *plr* open reading frame (ORF) at bp 420. Therefore any potential transcription of *plr* was interrupted by the Ω Km^r cassette. The cassette was originally from the plasmid pHP45Ω-Km, which is a derivative of the streptomycin resistant (Sm^r) interposon of plasmid pHP45Ω (Fellay et al., 1987). The Sm^r gene had been replaced with the Km^r gene of Tn5 to yield pHP45Ω-Km. The Km^r gene of the Ω cassette is flanked by transcriptional and translational terminators and the gene product is functional in both *E. coli* and streptococcal hosts.

Electroporation of DNA into group A strain 64/14. DNA was electroporated into strain 64/14 following the method of Simon and Ferretti (Simon and Ferretti, 1991) or a similar protocol by M. Caparon, St. Louis, Mo. (personal communication). A 5 ml starter culture of THY and 20 mM

glycine was grown as a standing culture at 37° C, ON. The culture was then diluted with the same medium so that the initial optical density (O.D.) was 0.06 to 0.08 when measured at an absorbance of 600_{nm}. The culture was incubated for 1 to 2 hrs at 37° C until the $O.D._{600nm}$ was approximately 0.20. Bacteria were pelleted by centrifugation at 14° C, suspended in 5 ml of the spent culture media, and then heat shocked for 9 min at 43° C. Cells were washed two times in 15% glycerol and suspended in a final volume of 1 ml with 15% glycerol. Between one and five micrograms of DNA and 100 µl of cells were added to pre-chilled electroporation cuvettes, 0.5 cm slit width (Biorad). The cells were subjected to a single pulse of 1.75 kV, 400 ohms. The cuvettes were subsequently kept on ice for 45 min, the bacterial suspension was diluted in 10 ml fresh THY broth, and then was incubated at 37° C for 1 hr. Bacteria were pelleted by centrifugation, suspended to 0.5 ml in THY broth and plated on THY agar containing 500 µg/ml kanamycin.

Both circular and linear pRL027 DNA were used in electroporation experiments. Linear DNA was prepared by either digesting the plasmid at the unique *N*co I restriction site, or alternatively by amplifying a 3.3 kb DNA fragment consisting of the *plr* ORF and the Ω cassette of pRL027 by PCR. The oligonucleotide primers RL22 5'-GTTAATACCAATAACTAC CATGGGCC-3' and RL21 5'-CGGGAGCTAATTATTTAGCAATTTTTGCG-3' were complementary to the 5' and 3' ends, respectively, of the *plr* ORF and were used in the PCR.

Protein purification. Streptococcal Plr (sPlr) was isolated from a mutanolysin extract of strain 64/14 prepared by a modification of a previously described protocol (Broder et al., 1991). Bacteria were pelleted by centrifugation, washed 3 times with phosphate buffered saline (PBS), pH 7.4 and suspended in PBS containing 30% raffinose (to prevent cell lysis), 1 mM PMSF, and 1 mM TLCK. Mutanolysin (Sigma Chemical Company, St. Louis, Mo.) was added at a concentration of 180 units per gram wet weight of bacterial pellet to degrade the peptidoglycan cell wall. The suspension was mixed gently for 2 h at 37° C to release cell wall-associated components. Protoplasts were pelleted by centrifugation, and the supernatant was filtered through a 0.2 µm filter. Ammonium sulfate was added to the filtrate to 60% saturation and stirred slowly overnight at 4° C. Precipitated proteins were removed by centrifugation at 15,000 X g for 10 min at 4° C. The remaining supernatant was dialyzed extensively in PBS and contained primarily sPlr.

Recombinant Plr (rPlr) was separated from the majority of *E. coli* proteins as described previously (Lottenberg et al., 1992a). Briefly, ammonium sulfate was added to a soluble lysate of *E. coli* χ6060 (pRL024) to 55% saturation, and stirred gently overnight at 4° C. Precipitated proteins were pelleted by centrifugation and rPlr remained predominantly in the supernatant.

Streptococcal cytoplasmic GAPDH (sGAPDH) was purified from strain 64/14 protoplasts generated by the mutanolysin treatment described above. Protoplasts were washed three times with 10 mM potassium phosphate, pH 6.8 and lysed by two passages through a French pressure cell. Unlysed protoplasts were removed by centrifugation at 8,000 X g for 10 minutes and the resulting supernatant was subjected to ultracentrifugation at 30,000 X g for 30 min to remove the remaining insoluble material. sGAPDH was purified from the soluble extract by NAD^+ affinity chromatography (Comer et al., 1975). Briefly, NAD^+-agarose (Sigma) was hydrated in 10 mM potassium phosphate buffer, pH 6.8, washed extensively, and loaded into a chromatography column. Protein extracts were incubated in the column matrix for 1.5 h at room temperature (RT) by end-over-end rotation. The matrix was washed extensively with phosphate buffer to remove unbound proteins. Bound proteins were eluted from the column by the addition of 10 mM NAD^+ (Sigma) to the phosphate buffer.

The 41-kDa protein(s), sPlr/sGAPDH, used for amino acid sequencing and amino acid composition analysis was isolated from a whole cell extract of strain 64/14. Bacteria were incubated with mutanolysin, and the mixture passed through a French pressure cell twice. Insoluble material was pelleted by centrifugation. The supernatant was loaded onto a NAD^+ affinity chromatography column (as described above) to isolate sPlr/sGAPDH.

SDS-PAGE and protein blotting. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to resolve proteins (Lammeli, 1970). Proteins were identified by staining with Coomassie brilliant blue. Proteins resolved on polyacrylamide gels were prepared for electrotransfer to nitrocellulose membranes by equilibrating the gel in 25 mM Tris-HCL, 0.2 M glycine, pH 8.0 with 20% vol/vol methanol. Proteins were electrotransferred from gels to nitrocellulose membranes using a Trans-Blot cell (Bio-Rad, Richmond, CA). Membranes were soaked in NET-gel (50 mM Tris, 150 mM NaCl, 5 mM EDTA, 0.05% vol/vol Triton X-100, and 0.25% wt/vol gelatin) prior to incubation with either primary antibody or [^{125}I]plasmin.

For the Western blots, polyclonal mouse anti-sPlr antibody (Broder et al., 1991) was used as primary antibody; followed with goat anti-mouse IgG (Organon Teknika Corp., Durham, NC) as secondary antibody which was detected with ^{125}I labeled protein G (Sigma).

Human glu-plasminogen (American Diagnostica, Inc., Greenwich, CT) was converted to plasmin using urokinase (Sigma) as the plasminogen activator (Broder et al., 1991) immediately prior to incubation with blots. Plasmin ligand blots were performed by incubating approximately 50,000 counts per minute of labeled plasmin per ml NET-gel with nitrocellulose membranes containing proteins of interest for 1 hr at RT. Membranes were then washed three times with NET-gel and exposed to autoradiography film overnight at -70° C.

Radiolabeled proteins. Protein G and plasminogen were radiolabeled with [^{125}I]Na (Amersham, Arlington Heights, IL) by a lactoperoxidase reaction using enzymobeads (Bio-Rad), and labeled proteins were separated from free label by gel filtration using a PD-10 column (Pharmacia Biotech Inc., Piscataway, NJ).

Peptide map analysis. Peptide maps were generated for sPlr, sGAPDH, and rPlr with V8 protease following the method of Cleveland (Cleveland et al., 1977). Purified proteins were electrophoresed on a SDS-10% polyacrylamide gel. Protein bands were visualized by staining with Coomassie brilliant blue, and the 41-kDa proteins were excised from the gel. Gel slices were incubated for 30 minutes in buffer (0.125 M Tris-HCL, 0.1% wt/vol SDS, 1 mM EDTA, pH 6.8). Gel slices were loaded into the wells of a SDS-polyacrylamide gel (4% stacking gel and a 15% separating gel) and overlaid with buffer containing 20% vol/vol glycerol and bromophenol blue. Five micrograms (15 U/mg) of V8 protease (Calbiochem-Novabiochem Corporation, San Diego, CA) in 10% vol/vol glycerol were added to each well. Samples were concentrated in the stacking gel by electrophoresis, and the digest was allowed to proceed for 3 h at RT before resuming electrophoresis. Following electrophoresis, proteins were visualized by staining with Coomassie brilliant blue.

Amino acid sequencing. The NH_2-terminal amino acid sequence had previously been determined for sPlr (Lottenberg et al., 1992b). The 41-kDa protein isolated from the whole cell extract and rPlr were electrophoresed on SDS-polyacrylamide gel and electrotransferred onto an Immobilon PVDF membrane (Millipore Corp., Lakeland, FL) in the presence of 10 mM MES, pH 6.0 in 20% methanol vol/vol. Proteins were then subjected to microsequencing using automated Edman chemistry at the Interdisciplinary Center for Biomedical Research (ICBR) Protein Chemistry Core Laboratory at the University of Florida, Gainesville.

Amino acid composition. The amino acid composition was determined for sPlr/sGAPDH and rPlr. Proteins were electrophoresed on SDS-polyacrylamide gel and then electrotransferred onto Immobilon PVDF membranes. The 41-kDa proteins were visualized with Coomassie brilliant blue and excised from the membrane. Amino acid composition was performed by the ICBR Protein Chemistry Core Laboratory, University of Florida, Gainesville. Proteins were hydrolyzed in 6N HCL with 1% phenol for 22 hr at 110° C, and amino acid composition was determined using the sodium buffer system with a Beckman System 6300 high performance analyzer.

GAPDH enzymatic assays. Purified proteins were assayed for GAPDH enzymatic activity following the protocol of Ferdinand (Ferdinand, 1964). Purified proteins in 50 µl

volumes were added to 100 µl of 20 mM DL-glyceraldehyde-3-phosphate (DL-GAP), 100 µl of 10 mM NAD^+, and 750 µl of reaction buffer (40 mM triethanolamine, 50 mM Na_2HPO_4, and 5 mM EDTA, pH 8.6). Negative control assays were performed as above without the addition of DL-GAP. The reduction of NAD^+ to NADH was monitored spectrophotometrically at an absorbance of 340 nm, and absorbances were recorded at 20 sec intervals for 4 min using a Beckman model DU-70 spectrophotometer (Beckman Instruments, Inc., Fullerton, CA). Absorbances were converted to micromoles NADH using the molar absorption coefficient of 6.22×10^{-3} (Horecker and Kornberg, 1948), and protein concentrations were determined using a bicinchoninic acid protein assay (Pierce, Rockford, Ill.) to calculate specific activities expressed as µM NADH min^{-1} mg^{-1}.

DNA hybridization studies. DNA hybridization was performed by the method of Southern (Maniatis et al., 1989). Chromosomal DNA was isolated by the method outlined by Caparon and Scott (Caparon and Scott, 1991), and some preparations were further purified on CsCl gradients. Five micrograms of genomic DNA were used per restriction enzyme digest. For strain 64/14, digested DNA was separated by electrophoresis in duplicate 0.7% wt/vol agarose gels and transferred to nylon membranes by a capillary blot procedure outlined in the manufacturer's instructions (Gene Screen Plus, Dupont Boston, MA). The DNA probe consisted of the 1-kb *plr* open reading frame (ORF) which was amplified by PCR

using the plasmid pRL024 as DNA template. The probe was labeled with [^{32}P]dCTP (Amersham, Corp., Arlington Heights, Ill.) using a random priming kit (United States Biochemical Corp., Cleveland, Ohio). The probe was then incubated with the membranes in the presence of 10% wt/vol dextran sulfate, 50% vol/vol formamide, and 0.5% wt/vol SDS for 18 h at either RT (low stringency) or 55° C (high stringency). Membrane washes were consistent with hybridization temperatures of RT or 55° C. Reactive bands were visualized by autoradiography. Southern blots of the 19 other streptococcal isolates were performed by the same method but with a hybridization temperature of 42° C and the washes were performed at 65° C.

Results

Immunochemical analysis and plasmin binding activity of sPlr, sGAPDH, and rPlr. I first examined fractions of total strain 64/14 proteins with anti-sPlr polyclonal antibody to identify proteins immunologically related to Plr. The antibody detected sPlr in the mutanolysin extract and an immunologically reactive protein band also migrating at ~41 kDa in the cytoplasmic fraction as shown in figure 1-1. There do not appear to be any other cross-reactive proteins in either the soluble cytoplasmic lysates or the mutanolysin extract. Additionally, the antibody did not detect any

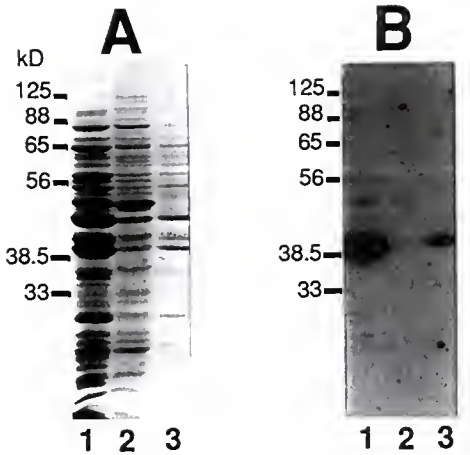


Figure 1-1. Anti-sPlr antibody reactivity of strain 64/14 lysate fractions. Samples were electrophoresed on triplicate reducing SDS-10% polyacrylamide gels. One gel was stained with Coomassie brilliant blue to visualize proteins (A) and the proteins on other gels were electrotransferred to a nitrocellulose membrane, blocked, and reacted with mouse anti-sPlr polyclonal antibody raised against mutanolysin extracted sPlr and detected with secondary antibody and [^{125}I]protein G as described in the Materials and Methods (B). Lanes 1 contain soluble cytoplasmic lysate, lanes 2 contain the insoluble fraction, and lanes 3 contain mutanolysin extracted proteins.

cross-reactive proteins in the insoluble fraction containing membranes.

To determine if the anti-sPlr immunoreactive protein in the cytoplasmic fraction was a streptococcal GAPDH (sGAPDH) and to examine the relatedness of this protein to sPlr and rPlr, these proteins were purified as described in Materials and Methods and resolved on triplicate SDS-PAGE. One gel was stained with Coomassie brilliant blue to visualize proteins (figure 1-2A) and revealed that the predominant band migrated at ~41 kDa for each preparation. The proteins resolved on the other two gels were electrotransferred to nitrocellulose membranes, and probed with either ^{125}I-labeled plasmin or polyclonal anti-sPlr antibody. As shown in figure 1-2B, all three 41-kDa proteins reacted with anti-sPlr antibody which indicates cross-reactive epitope(s). Additionally, each of the proteins bound radiolabeled plasmin (figure 1-2C) demonstrating a functional similarity among them. The three protein samples were then examined at the primary amino acid level to further determine the extent of similarity among them.

Analysis of Protein Composition. To determine if homologies among sPlr, sGAPDH, and rPlr extended to amino acid residue position, peptide maps were generated by V8 protease. V8 protease cleaves peptide bonds on the carboxylic side of aspartate and glutamate. Digested proteins were separated by SDS-PAGE and visualized by

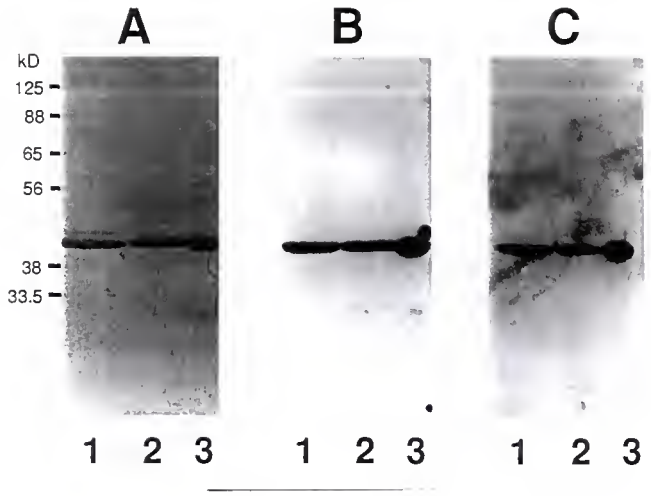


Figure 1-2. Anti-sPlr antibody reactivity and plasmin binding ability of purified proteins. Samples were electrophoresed on triplicate reducing SDS-10% polyacrylamide gels. One gel was stained with Coomassie brilliant blue to visualize proteins (A). The proteins resolved on the other two gels were transferred to nitrocellulose membranes, blocked, and reacted with either mouse anti-sPlr antibody (B), or with [^{125}I]plasmin (C). Lanes 1 contain streptococcal Plr (sPlr), lanes 2 contain streptococcal cytoplasmic GAPDH (sGAPDH), and lanes 3 contain recombinant Plr (rPlr).

staining with Coomassie brilliant blue. Digests of sPlr, sGAPDH, and rPlr yielded identical size peptides (figure 1-3) revealing conservation of acidic residues throughout the sequence of the three proteins, as well as approximately the same number of amino acids between them.

NH_2-terminal amino acid sequencing of rPlr and sGAPDH/sPlr (from a whole cell preparation) was performed to establish if homologies extended to the conservation of amino acid sequence. Analysis of rPlr and sGAPDH/sPlr revealed the identical NH_2-terminus as that of the previously determined sequence of sPlr. Unambiguous sequence was obtained for all samples. The amino acid sequence of sGAPDH/sPlr was as follows: V V K V G I N G F G R I G R L A F R R I. Valine was also the NH_2-terminal amino acid for sPlr whereas the recombinant protein contained an approximately 50:50 mixture of protein with and without the NH_2-terminal methionine. Although the NH_2-termini were identical, it was possible that amino acid differences exist elsewhere in the proteins.

In addition to the NH_2-terminal identity of the three samples, amino acid composition analysis of rPlr and the sPlr/sGAPDH mixture revealed no significant differences in overall protein composition between samples (Table 1). This further indicated the relatedness of the proteins at the amino acid level. However, amino acid composition analysis

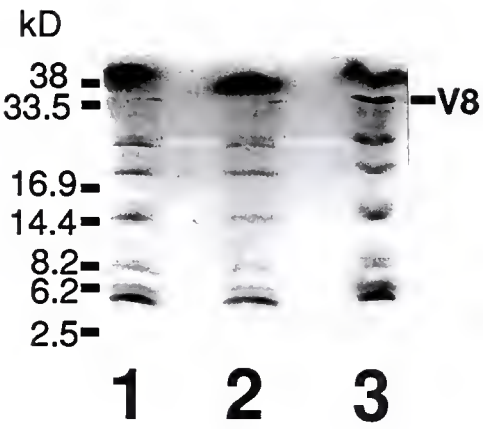


Figure 1-3. Peptide map analysis of purified proteins generated by staphylococcal V-8 protease digestion. Purified proteins were prepared as described in Materials and Methods and digested with V-8 protease in the gel. Peptides were separated on a reducing SDS-15% PAGE and identified with Coomassie brilliant blue. The arrow indicates the V-8 protease. Lane 1 contains sPlr, lane 2 contains sGAPDH, and lane 3 contains rPlr.

Table 1. Amino acid composition comparison of sGAPDH/Plr, rPlr, and SDH.

	Number of amino acid residues			
amino acid	rPlr	sGAPDH/ Plr	Plr predicted a.a. seq.	SDH[a]
Ala	25.3	29.6	33	38.1
Arg	19.8	27.2	13	15.5
Gly	35.5	38.7	33	36.6
His	8.0	7.8	7	7.2
Ile	19.4	16.6	21	22.4
Leu	24.5	24.4	22	23.4
Lys	19.7	19.8	20	21.4
Met	3.8	2.1	8	1.8
Phe	12.7	12.0	12	13.8
Ser	20.7	21.1	15	16.8
Val	25.3	24.1	35	36.5
Asn/Asp	49.0	39.2	42	43.3
Gln/Glu	30.5	32.6	27	29.9
Pro	5.5	8.9	10	13.6
Thr	27.7	22.8	26	27.0
Tyr	7.7	8.3	7	9.1

[a]Pancholi and Fishetti, 1992.

may not be sensitive enough to detect small quantitative differences for some amino acids.

These procedures detected no differences among the sPlr, sGAPDH, rPlr indicating that they are structurally similar proteins. The analysis was extended to functional properties of GAPDH to further examine the ~41 kDa proteins.

NAD binding and GAPDH enzymatic activity. NAD^+ is reduced to NADH during the oxidative phosphorylation of glyceraldehyde-3-phosphate into 1,3-bisphosphoglycerate by GAPDH. GAPDH contains a NAD^+ binding domain in the NH_2-terminal half of the protein (Harris and Waters, 1976). Streptococcal GAPDH (sGAPDH) was purified from a cytoplasmic extract prepared from strain 64/14 by NAD^+ affinity chromatography (figure 1-4B). In this experiment, a ~41 kDa protein was the predominant protein in the NAD^+ elution fractions and was confirmed to be a GAPDH by exhibiting glycolytic enzyme activity (see below). The identical protocol used to isolate sGAPDH was also utilized to demonstrate that sPlr was a NAD^+ binding protein. A mutanolysin extract of strain 64/14 was applied to the affinity column and sPlr was the predominant protein eluted with 10 mM NAD^+ as shown figure 1-4A. Similarly, rPlr was affinity purified from a soluble lysate of *E. coli* χ6060 (pRL024) (data not shown). Therefore sPlr, sGAPDH, and rPlr share the GAPDH characteristic of having functional NAD^+ binding domains.

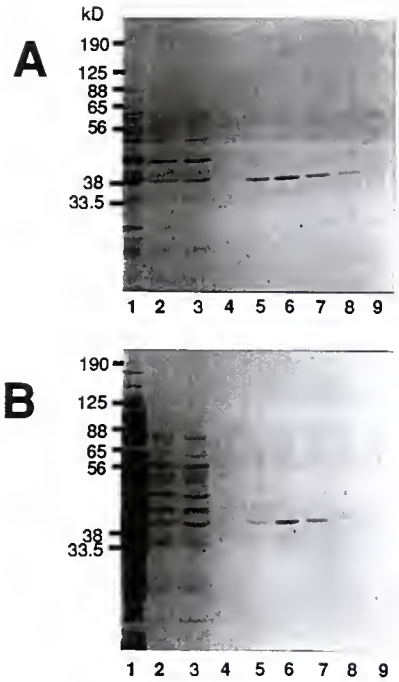


Figure 1-4. NAD^+ affinity chromatography purification of the 41-kDa proteins. Mutanolysin extracted proteins from strain 64/14 (A) or soluble cytoplasmic material from strain 64/14 (B) were applied to a NAD^+-agarose affinity column and bound proteins eluted from the column with 10 mM NAD^+. Proteins were resolved by SDS-10% polyacrylamide gel electrophoresis and identified with Coomassie brilliant blue. Lanes 1 contain starting material applied to the column. Lanes 2 and 3 contain wash fractions, and lanes 4-8 contain elution peak fractions by the addition of 10 mM NAD^+ to the wash buffer.

The three purified 41-kDa proteins were assayed for glycolytic enzyme activity to verify that they were functional GAPDH enzymes. Analysis of purified sPlr, sGAPDH, and rPlr yielded specific activities of 62.6, 153.1, and 206.3 μM NADH min^{-1} mg^{-1}, respectively. There was never any detectable spontaneous conversion of NAD^+ to NADH without the addition of DL-GAP to the reaction mixtures. Therefore the three purified proteins demonstrate functional GAPDH enzymatic activity and have specific activities within the range reported for other GAPDH molecules of prokaryotic origin (Branlant et al., 1983). Based on both the structural and functional data, it appears that sGAPDH and sPlr are the same primary protein in strain 64/14 and that this protein is identical to recombinant Plr.

DNA hybridization studies. Organisms which express only a single GAPDH may possess more than one *gap* gene. Therefore, DNA hybridization analysis by the method of Southern was performed on strain 64/14 chromosomal DNA using a *plr* probe to examine whether the genome contained a single or multiple copies of *plr*. To also identify possible related genes or psuedogenes both hybridization with the *plr* probe and washes of the membrane were done under conditions of low stringency at room temperature (RT), to allow for maximum mismatch detection using this technique (figure 1-5A). Conditions of high stringency at 55° C, were performed for comparison (figure 1-5B). In lanes containing chromosomal

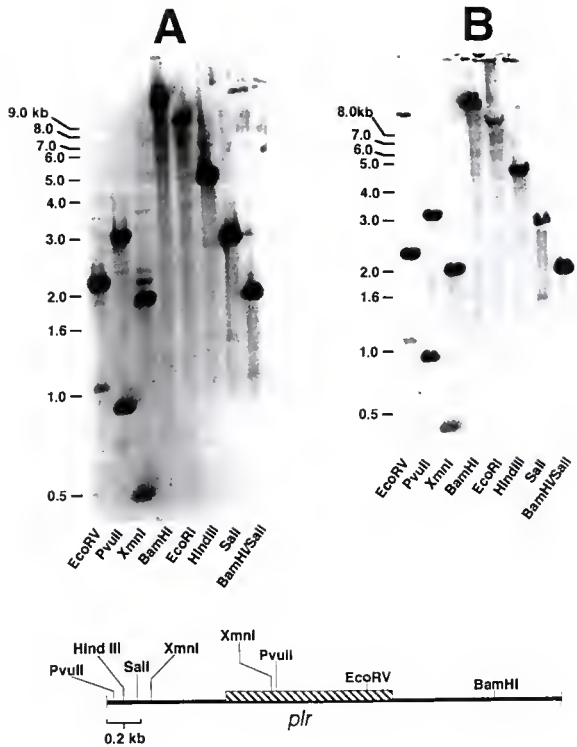


Figure 1-5. DNA hybridization analysis of strain 64/14 chromosomal DNA to determine gene copy number of *plr*. DNA was digested with the restriction enzymes indicated in the figure, electrophoresed on duplicate 0.7% agarose gels, and transferred to nylon membranes. The membranes were then reacted with [^{32}P]dCTP labeled probe consisting of the *plr* ORF, washed, and subjected to autoradiography. One autoradiograph shows the overnight hybridization and washes performed at RT (A). The other autoradiograph shows the hybridization and washes performed at 55°C (B). The diagram indicates relevant restriction enzyme sites located on the 2.7-kb DNA fragment cloned from strain 64/14 which harbors *plr* and flanking regions.

DNA digested with restriction enzymes which cut once within *plr* (*Eco*RV, *Pvu*II, and *Xmn*I), the probe hybridized with two fragments at both RT and 55° C. The probe hybridized with a single fragment in lanes containing DNA digested with restriction enzymes which cut outside of *plr* (*Bam*HI, *Eco*RI, *Hind*III, and *Sal*I). These results are consistent with a single copy gene. The *Bam*HI/*Sal*I double digest yielded a single 2 kb fragment that is too small to harbor more than one copy of *plr* (see restriction map of figure 1-5). Therefore, *plr* is a single copy gene in group A strain 64/14.

To confirm that a single copy of *plr* is typical for group A streptococci, a series of nineteen other group A streptococcal strains isolated from both throat and blood cultures (Wang et al., 1994) was examined by DNA hybridization. Chromosomal DNA was digested with *Bam*HI, *Sal*I, or a *Bam*HI/*Sal*I double digest. The hybridization pattern using the *plr* probe for all nineteen strains was identical to that of strain 64/14 as shown for the four representative strains SHS 7, SHS 9, SHS 17, and 230041 in figure 1-6. The *Bam*HI and *Sal*I digests yielded a 10 kb and a 3.0 kb size fragment, respectively, and the *Bam*HI/*Sal*I double digests yielded a single 2.2 kb fragment for all isolates. These results indicated that all strains tested contained a single copy of *plr* and suggested that this may be typical for group A streptococci.

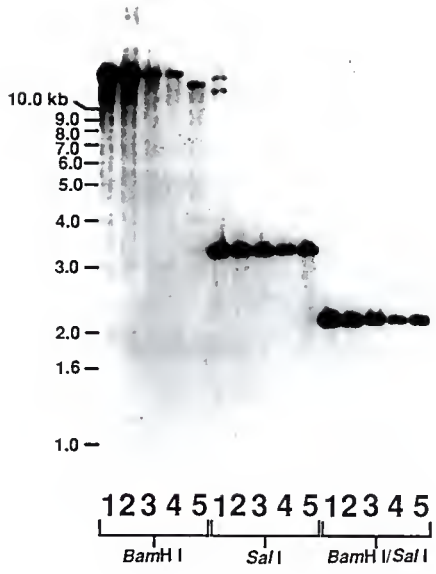


Figure 1-6. DNA hybridization analysis to determine gene copy number of *plr* in group A streptococcal clinical isolates. Strain 64/14 and four representative strains of 19 strains tested, are shown in this figure. Chromosomal DNA was digested with the restriction enzymes *BamHI*, *SalI*, or a *BamHI*/*SalI* digest; electrophoresed on a 0.7% agarose gel; and transferred to a nylon membrane. The membrane was then reacted with a [^{32}P]dCTP labeled probe consisting of the *plr* ORF. Following overnight hybridization at 42°C, the membrane was washed, and reactive bands visualized by autoradiography. Lanes 1 strain 64/14, lanes 2 SHS-7, lanes 3 SHS-9, lanes 4 SHS-17, lanes 5 strain 230041.

Strategy for insertional inactivation of *plr*. The goal of this experiment was to generate an isogenic mutant of strain 64/14 in *plr* by insertionally inactivating the gene with a DNA cassette containing an antibiotic resistance marker (figure 1-7A). Linear pRL027 DNA, containing the *plr* ORF interrupted by the Ω cassette, was electroporated into strain 64/14, and bacteria were plated on either blood agar or THY agar plates containing 500 µg/ml kanamycin. Circular pRL027 was also electroporated in separate experiments as a control to verify that strain 64/14 was transformable and that pRL027 could integrate into the chromosome (figure 1-7B). In contrast to successful transformation with circular DNA, repeated attempts failed to produce a single kanamycin resistant colony using the linear DNA. The plasmid contained over 1 kb of homologous DNA both 5' and 3' to the Ω cassette which should be of sufficient length to allow a crossover event to occur. Furthermore hybridization analysis of chromosomal DNA from an isolate transformed with circular pRL027 using both *plr* and Ω cassette gene probes indicated that the plasmid had integrated directly downstream to the *plr* gene (data not shown). These results suggested that the *plr* gene may be essential for viability in strain 64/14.

Growth media requirements of strain 64/14. By providing a carbon source which could potentially be utilized for the generation of ATP by enzymes that function in the glycolytic

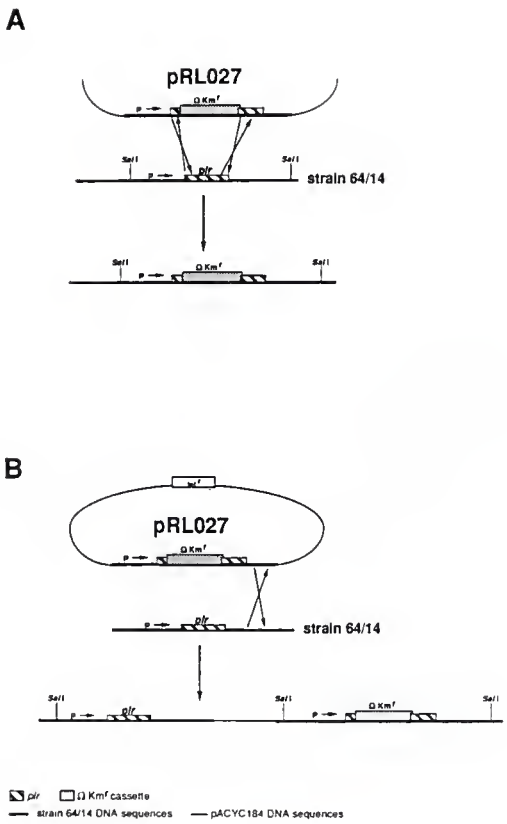


Figure 1-7. Potential recombination events resulting from electroporation of plasmid pRL027 into strain 64/14. Electroporation of linear pRL027 would potentially yield a double crossover event by homologous recombination, indicated by the arrows, resulting in inactivation of *plr* (A). Circular pRL027 integrated via a single crossover event occurring downstream of *plr* on the chromosome,and therefor retained a full-length copy of *plr* (B).

pathway subsequent to the reaction involving GAPDH, it may be possible to eliminate the need for this enzyme, and thus provide an opportunity to successfully inactivate the *plr* gene. Group A streptococci can be grown in culture using a chemically defined medium (CDM) (Van Derijn et al., 1980) containing 1% wt/vol glucose as the primary carbon source. Strain 64/14 was inoculated into a modified CDM prepared with 0.5% wt/vol succinate and 0.5% vol/vol glycerol. There was no visible growth after several days of incubation at 37° C. The bacteria grew only in succinate/glycerol CDM to which glucose was added. Attempts at growing the streptococci in CDM containing either 1% wt/vol acetate or 1% wt/vol pyruvate instead of glucose were also unsuccessful indicating that, of the reagents used in these experiments, glucose is the only carbon source which can be transported into and/or utilized by the glycolytic pathway of strain 64/14. This result was consistent with *plr* being an essential gene.

Discussion

Our laboratory previously demonstrated that group A streptococci bind plasmin with high affinity (Broeseker et al., 1988). A plasmin binding protein, sPlr, has been isolated from mutanolysin extracts of group A strain 64/14 (Broder et al., 1991). The gene encoding this candidate plasmin receptor has been cloned, sequenced, and expressed in

Escherichia coli. The entire deduced and partially experimentally determined amino acid sequence of sPlr showed significant homology to glyceraldehyde-3-phosphate dehydrogenases of both prokaryotic and eukaryotic origins (Lottenberg et al., 1992a). GAPDH is an enzyme of the glycolytic pathway that has been extensively characterized for a variety of organisms. As expected, it is usually localized to the cytoplasm or to specific organelles within a cell, although there are reports of surface localization for GAPDH in several organisms (Fernandes et al., 1992; Goudot-Crozel et al., 1989).

In this study the relationship between sPlr and cytoplasmic GAPDH from strain 64/14 was examined. GAPDH is a tetrameric enzyme of the glycolytic pathway responsible for the phosphorylation of glyceraldehyde-3-phosphate to generate 1,3-bisphosphoglycerate (Harris and Waters, 1976). NAD^+ binds to GAPDH at a specific site and serves as an electron acceptor for the substrate during the reaction. By using NAD^+ affinity chromatography, I was able to isolate streptococcal GAPDH (sGAPDH) from a cytoplasmic extract of strain 64/14. A single protein, migrating at ~41 kDa on SDS-PAGE was eluted from the affinity column with excess free NAD^+. This 41-kDa protein was compared with both Plr isolated from mutanolysin extracts of group A strain 64/14 (sPlr) and purified recombinant Plr (rPlr) to evaluate the relatedness of the proteins. Amino acid analysis of sGAPDH, sPlr, and rPlr revealed identical NH_2-terminal amino acid

sequences for the three proteins by Edman degradation. This amino acid sequence was in complete agreement with the predicted amino acid sequence of Plr. V8 protease digestion of the three purified proteins yielded peptides of equivalent size when identified by SDS-PAGE indicating conservation of residues throughout the proteins. In addition to these amino acid homologies among the proteins, polyclonal antibody raised against sPlr also recognized both rPlr and the sGAPDH on Western blot analysis. Furthermore, no cross-reacting proteins were detected other than sGAPDH and sPlr in a strain 64/14 soluble lysate and mutanolysin extract, respectively. No differences in primary protein structure of sPlr, sGAPDH, and rPlr were detected. Although anti-Plr polyclonal antibody recognizes only a single protein in group A streptococcal lysates, GAPDHs from a single organism may differ significantly in amino acid composition and contain different antigenic epitopes. For example, *Trichoderma koningii* expresses two GAPDH isozymes, GAPDH I and GAPDH II. The glycolytic activity of GAPDH I is inhibited by koninigic acid whereas GAPDH II is resistant. Analysis of the NH_2-terminal amino acid sequences revealed only 70% similarity to each other, and antisera against GAPDH II are only weakly cross reactive with GAPDH I (Sakai et al., 1990).

The streptococcal proteins were functionally alike as well. sPlr, sGAPDH, and rPlr each bound to a NAD^+ affinity column, possessed GAPDH enzymatic activity, and demonstrated plasmin binding activity using a ligand blot assay. Based on

these structural and functional identities, we conclude that sPlr and sGAPDH are the same primary protein. Furthermore, using the techniques in this study, Plr/GAPDH cannot be differentiated from rPlr, which was expressed in *E. coli* from recombinant *plr*.

Prokaryotic and eukaryotic organisms may possess single or multiple GAPDH genes. In this report DNA hybridization studies revealed that strain 64/14 harbors a single copy of *plr*, a gene encoding a streptococcal GAPDH. Furthermore, studies of 19 group A streptococcal clinical isolates yielded hybridization patterns identical to those of strain 64/14 indicating that these strains contained a single copy of *plr* as well. These results suggest that group A streptococci may typically possess a single gene encoding Plr. However, the possibility remains of a second, highly divergent *gap* gene which is present in the streptococcal chromosome but is not detectable by DNA hybridization analysis using a *plr* probe. This situation would be analogous to *E. coli* which has at least two GAPDH genes (Alefounder and Perham, 1989). The *gap* A appears to be similar to GAPDHs of eukaryotic origins while the *gap* B has highest DNA homology with other prokaryotic GAPDH genes. Only expression of *gap* A has been reported. In contrast, *Sacharomyces cerevisiae* has three GAPDH genes, all of which are expressed (Holland, 1983).

There are organisms which utilize one GAPDH for cytoplasmic functions while another GAPDH is targeted for specific organelles (Michels et al., 1991). In the protozoa

Trypanosoma brucei, one GAPDH isozyme is located in glycosomes, specialized glycolytic organelles containing the first seven enzymes of the glycolytic pathway. A second GAPDH isozyme resides solely in the cytoplasm. The glycosomal GAPDH contains amino acid substitutions throughout the protein relative to the cytosolic GAPDH as well as several additional amino acids at the C-terminus which are thought to be involved in targeting the protein to the glycosomes. The genes encoding these GAPDHs have been cloned and the predicted amino acid sequences are 55% identical. DNA hybridization studies indicated there are two genes in tandem that could potentially express the glycosomal form, whereas only a single gene was detected for the cytosolic enzyme (Michels et al., 1986). *Leishmania mexicana* also has two GAPDH isozymes, a glycosomal and a cytosolic form. The *gap* gene arrangement of *L. mexicana* is similar to *T. brucei*, and the predicted amino acid sequences of the two GAPDHs are only 55% identical (Hannaert et al., 1992). However, in the present study there was no experimental evidence for either a second GAPDH product in strain 64/14 or more than one homologous gene encoding Plr. The DNA sequence of *plr* does not contain the putative Gram-positive membrane anchor motif sequence nor does it reveal a protein-secretion signal sequence (Lottenberg et al., 1992a). Additionally, Plr was not detected by Western blot analysis in the insoluble fraction of a strain 64/14 lysate (figure 1-1B, lane 2)

indicating that Plr may not be associated with the bacterial membrane.

GAPDHs and putative GAPDHs have been reported to be localized on the surface of several organisms. The yeast *Kluyveromyces marxianus* flocculates when subjected to heat stress. The accumulation of a 37-kDa protein in the yeast cell wall during flocculation has been described (Fernandes et al., 1992). NH_2-terminal amino acid sequencing was determined for V-8 protease generated peptides of the cell wall purified protein which revealed over 80% identity to one of the *Sacharomyces cerevisiae* GAPDH molecules. Interestingly, the cell wall-associated GAPDH homologue is glycosylated, as demonstrated by its susceptibility to cleavage by endoglycosidase-H, whereas the cytosolic form is not. Although post-translational modifications occur less frequently in prokaryotes, analysis for potential post-translational modifications of Plr/GAPDH has not yet been performed, but could yield clues regarding putative secondary functions and/or localization of Plr/GAPDH.

GAPDH has also been reported to be localized on the surface of *Schistosoma mansoni*. Indirect immunofluorescence was used to identify GAPDH on whole *S. mansoni* and Western blot analysis identified GAPDH in isolated tegument preparations using antibody raised against the cytosolic GAPDH (Goudot-Crozel et al., 1989). Furthermore, antisera from patients who had severe *S. mansoni* infections contained low titers of antibody directed against *S. mansoni*, whereas

those who were less susceptible to the blood fluke had higher titers of antibody that reacted with the 37-kDa protein. In addition to GAPDH, another glycolytic enzyme, triose phosphate isomerase has been reported to be exposed on the *S. mansoni* surface (Harn et al., 1988). The potential functions of glycolytic enzymes on the surface of this organism have not yet been defined and the in vivo significance remains to be elucidated.

Recently Pancholi and Fishetti reported a surface GAPDH molecule, SDH, from group A streptococci (Pancholi and Fischetti, 1992). Using methodology similar to the isolation of Plr, SDH was purified from cell wall extracts of group A streptococci that were prepared using the muramidase lysin. SDH was reported to bind to fibronectin, lysozyme, actin, and myosin on a ligand blot assay; and to have ADP-ribosylation activity in vitro (Pancholi and Fischetti, 1993). The NH_2-terminal amino acid sequence of SDH is identical to the NH_2-terminal amino acid sequence of Plr with the exception of an alanine^{33} in SDH in contrast to an arginine^{33} in Plr.

In this report, NAD^+ affinity chromatography was used to purify a 41-kDa protein isolated from a whole cell preparation of strain 64/14. The NAD^+ purified protein, as well as rPlr, were subjected to acid hydrolysis to discern amino acid composition of the proteins. The differences in amino acid composition between sPlr/sGAPDH and rPlr were no greater than differences between the deduced amino acid sequence of rPlr and the experimentally determined amino acid

composition of rP1r. Furthermore these differences are no greater than those of the reported amino acid composition of SDH compared to that of the sP1r/GAPDH and rP1r proteins (see Table 1). The amino acid composition analysis of SDH detected 1.8 methionine residues compared to 7 residues in the *Bacillus stearothermophilus* GAPDH (Pancholi and Fischetti, 1992). The authors speculated that a family of structurally diverse GAPDH molecules may be expressed on the surface of group A streptococci which may differ from the GAPDH(s) utilized for glycolysis found in the cytoplasm. Our amino acid composition analyses also detected similar low numbers of methionine residues; however, the predicted amino acid sequence of P1r indicates 7 methionines in the mature protein which is consistent with the *B. stearothermophilus* GAPDH. Four of these seven residues have been confirmed experimentally in our laboratory by cyanogen bromide fragmentation of P1r and subsequent NH_2-terminal amino acid sequencing of these fragments (Lottenberg et al., 1992a). It appears that P1r and SDH are structurally similar proteins. It is not uncommon for small amino acid changes in GAPDH to occur among different strains of the same species. For example, comparison of gap A nucleotide sequences of 13 *E. coli* strains and 16 *Salmonella* strains indicated 0.1 % and 1.1% amino acid differences, respectively, that had occurred among the strains. A larger number of the predicted amino acid substitutions resided in the NH_2-terminal portion of the

molecule comprising the NAD^+ binding domain of the molecule (Nelson et al., 1991).

To definitively assess the function of Plr as a plasmin receptor, the gene would be inactivated and the ability of the bacteria to capture surface bound plasmin would be compared to the wild-type strain. However, attempts at insertional inactivation of *plr* were unsuccessful using linearized plasmid pRL027 DNA, which contained a functional selectable marker inserted into the *plr* ORF. A double crossover of this DNA into the streptococcal chromosome would have replaced the wild-type copy of *plr*. The introduction of circular pRL027 into strain 64/14, which would integrate via a single crossover and thereby retain the wild-type copy of the gene, did yield kanamycin resistant transformants indicating that the construct could integrate into the chromosome and that the kanamycin resistance marker was functional in strain 64/14. Subsequent mutagenesis experiments (see Chapter 3) demonstrated that linear DNA can be successfully transformed into strain 64/14 and that the regions of DNA homology flanking the Ω cassette (300 bp 5' and 600 bp 3' to the cassette) were of sufficient length to allow the recombination events to occur. The integration of circular plasmid pRL027 into the chromosome eliminated the possibility that the Ω cassette which is flanked by transcription terminators was disrupting expression of potentially essential genes downstream of *plr*. Thus, these data suggested that *plr* may be an essential gene.

Taken together the data indicate that a candidate surface plasmin receptor, Plr, is the same primary protein as a GAPDH isolated from the streptococcal cytoplasm. The *plr* gene which expresses glycolytically active Plr resides as a single copy in the clinical group A strains tested in this study. The inability to successfully grow strain 64/14 in media containing carbon sources other than glucose suggests that Plr/GAPDH may be required for growth. Furthermore, the failure to successfully inactivate *plr* indicates that this *gap* may be an essential gene in group A streptococci.

CHAPTER 2
CHARACTERIZATION OF THE PLASMIN BINDING DOMAIN(S) OF PLR BY GENETIC MUTATION OF *plr*

Studies presented in the previous chapter demonstrate that Plr is a streptococcal glyceraldehyde-3-phosphate dehydrogenase (GAPDH) enzyme encoded by a single gene on the chromosome. Furthermore *plr* appears to be an essential gene in strain 64/14. Therefore one approach to evaluate the role of Plr as a plasmin receptor is to generate a non-plasmin binding Plr mutant that retains GAPDH enzymatic activity and then introduce that mutation(s) at the *plr* locus of strain 64/14. A series of genetic alterations of recombinant *plr* were constructed and the expressed products were analyzed for immunoreactivity with anti-Plr antibody, plasmin binding ability, and GAPDH enzymatic activity. The importance of a C-terminal lysine residue of Plr in the interaction with plasmin is clearly demonstrated in this chapter. Mutant Plr containing alterations of this C-terminal lysine retain GAPDH activity indicating that the two activities are at least partially separable. These mutant *plr* genes are therefore potential candidates for gene replacement of *plr* in strain 64/14.

Materials and Methods

Bacterial strains and growth conditions. The bacterial strains used in these studies were *E. coli* χ2602, *E. coli* χ6060 and *E. coli* DE3. The genotypes of *E. coli* χ2602 and *E. coli* χ6060 are listed in the Materials and Methods of Chapter 1. The genotype of *E. coli* DE3 is *end*A1, *hsd*R17 (r_k^-, m_k^+), *sup*E44, *thi*$^{-1}$, *rec*A1, *gyr*A96, *lac* [F' *pro*AB, *lac*I^q, ZΔM15::Tn10 (tet^r)] (Novagen, Inc., Madison, WI). Bacteria harboring plasmids were grown as shaking overnight cultures at 37° C in Luria both. Chloramphenicol, ampicillin, tetracycline, and kanamycin were used at concentrations of 30, 50, 34, and 10 μg/ml, respectively, where appropriate. Protein expression from pTrc99C and pMal-c2 derivatives were induced by the adding a final concentration of 10 mM IPTG to the Luria broth.

Construction of plasmids. The plasmids used in these studies are described in Table 2-1. DNA manipulations used in construction of plasmids was performed by standard methodology (Maniatis et al., 1989).

A series of 3' end deletions was generated in *plr* using exonuclease III by the protocol of Henikoff (Henikoff, 1987). Exonuclease III will degrade DNA which has a free overhanging 5' end or a blunt end, but not an overhanging 3' end. The plasmid pRL024 was digested with *Sph*I and *Bam*HI. Both restriction sites lie downstream of the *plr* ORF.

TABLE 2-1. Plasmids containing mutations of *plr*.

Plasmid	Vector	Insert Description	Marker[a]
pRL036	pACYC184	A 228 bp deletion of *plr* with 23 predicted vector encoded codons fused to the 3' end of the *plr* deletion. Exonuclease III treatment of pRL024 (see Materials and Methods) was used to generate the unidirectional deletion.	cm
pRL037	pACYC184	A 4 bp deletion of *plr* with 17 predicted vector encoded codons fused to the 3' end of the *plr* deletion. Exonuclease III treatment of pRL024 (see Materials and Methods) was used to generate the unidirectional deletion.	cm
pRL038	pACYC184	A 114 bp deletion of *plr* with 23 predicted vector encoded codons fused to the 3' end of the *plr* deletion. Exonuclease III treatment of pRL024 (see Materials and Methods) was used to generate the unidirectional deletion.	cm
pRL028	pTrc99c	The *plr-28* ORF has the terminal four amino acid codons of *plr* deleted by PCR mutagenesis of recombinant *plr*.	amp
pRL045	pTrc99c	The terminal codon of *plr* was changed from AAA encoding a lysyl residue to CTT encoding a leucine. The base pair changes are followed by a termination codon, TAA.	amp
pRL046	pTrc99c	The penultimate lysine codon of *plr*, AAA, was changed to a leucine codon, CTT. The terminal lysine codon was unaltered.	amp
pRL049	pTrc99c	A 99 bp 5' end deletion of *plr*. The protein product, Plr-49, is missing the NH_2-terminal 33 amino acids of the wild-type protein.	amp

TABLE 2-1.--Continued.

Plasmid	Vector	Insert Description	Marker[a]
pRL029	pACYC184	A 726 bp internal deletion of *plr* generated by utilizing *Bst*YI restriction enzyme sites. The expressed protein, Plr-29, is a fusion protein consisting of the NH_2-terminal thirty three amino acids and the C-terminal sixty amino acids of Plr.	tet
pRL042	pTrc99c	The third codon of *plr*, AAA encoding lysine was been changed to a glycine codon, GGT.	amp
pRL061	pTrc99c	A 39 bp 5' end deletion of *plr*. The product, Plr-61, is a NH_2-terminal truncation of Plr lacking 13 amino acids.	amp
pRL062	pTrc99c	A 15 bp 5' end deletion of *plr*. The product, Plr-62, is a NH_2-terminal truncation of Plr lacking 5 amino acids.	amp
pRL070	pMal-c2	The *plr* ORF was ligated in-frame with the *mal* E gene of pMal-c2 vector. When the expressed fusion protein, Plr-70, is cleaved with factor Xa eight predicted amino acids NH_2-terminal to methionine[1] of Plr.	amp
pRL071	pMal-c2	A 347 bp 5' deletion of *plr* was generated using PCR mutagenesis. The 0.7 kb amplified product was ligated into the pT7Blue T-vector (Novagen, Madison, WI). The 0.7 kb *plr* fragment was ligated into pMalc-2 vector resulting in an in-frame fusion of the *mal*E gene. When the expressed fusion protein, Plr-71, is cleaved with factor Xa there is one predicted amino acid in addition to amino acids 219-335 of Plr.	amp

[a]cm, chloramphenicol; amp, ampicillin; tet, tetracycline

TABLE 2-2. Oligonucleotide primers used for PCR mutagenesis of *plr*.

Plasmid	DNA sequence of oligonuceotides used to amplify *plr* inserts[a]
pRL033	RL22 5'-GTTAATACCAATAACTACCATGGGCC-3', RL33 5'CGCGGATCCAAGCTTCTAATTATTTAGCAATTTTTG CG-3'
pRL028	RL22, RL27 5'-CGGAAGCTTTATGCGAAGTAC-3'
pRL045	RL22, RL45 5'-CGCGGATCCTTAAAGAGCAATTTTTGC GAAGTACTCAAG-3'
pRL046	RL22, RL46 5'-CGCGGATCCTTATTTAGCAATAAGT GCGAAGTACTCAAG-3'
pRL049	RL49 5'-GGCCCATGGACCTTAGAGATCCAAATATG-3', RL21 5'-CGGGAGCTAATTATTTAGCAATTTTTGCG-3'
pRL042	RL42 5'-GGCCCATGGTAGTTGGAGTTGGTATTAAC GGTTTC-3', RL33
pRL061	RL61 5'-CGCCATGGGACGTCTTGCATTCCGCCGT-3', RL33
pRL062	RL62 5'-CGCCATGGGTATTAACGGTTTCGGTCGT-3' RL33
pRL070	RL36 5'-CCAGGAGCTGTGATAACAACCCCGGGGA TCCGCGC-3', RL33
pRL071	RL35 5'-GTTAATACCAACTTTAACTACCATGAATTC GGATCCGGC-3', RL33

[a]The forward primer is listed first. The plasmid pRL024 was used as DNA template for PCR reactions.

Exonuclease III was added to buffer containing linearized plasmid DNA. Aliquots were removed and the reactions terminated at 30 sec intervals for 10 min. Plasmid ends were blunt ended with S1 nuclease treatment followed by the addition of Klenow fragment and dNTPs. The plasmids were ligated and transformed into *E. coli* χ6060. Protein products expressed by transformants were examined by subjecting *E. coli* lysates to electrophoresis on polyacrylamide gels, which were stained with Coomassie brilliant blue to visualize the proteins, and for immunoreactivity with anti-Plr polyclonal antibody by Western blot analysis. Three plasmids with apparent 3' end deletions in *plr*, pRL036, pRL037, and pRL038, were chosen for further study. There was no stop codon introduced before ligation of digested plasmids, and therefore translation of *plr* mutations continued into vector derived-codons until a termination codon was reached. The predicted amino acid sequence derived from experimentally determined DNA sequence of the *plr* 3' end regions indicated that the expressed Plr mutations contained C-terminal amino acid residues encoded by vector derived sequences. The plasmids pRL036 and pRL038 have 228 and 114 base pairs (bp), respectively, deleted from the 3' end of wild-type *plr*. The resulting expressed proteins, Plr-36 and Plr-38, represent 76 and 38 amino acid deletions of the wild-type Plr C-terminus. There are predicted to be 23 amino acids fused to the C-terminal end of Plr-36 and Plr-38; the C-terminal five

residues consisting of asparagine, alanine, glycine, valine, and alanine. The plasmid pRL037 has 4 bp deleted from the 3' end of *plr*. Its product, Plr-37, has only the wild-type C-terminal lysine residue deleted. The C-terminus of this protein is fused to 17 vector-encoded amino acids which terminate with the predicted sequence tyrosine, tryptophan, alanine, alanine, and serine.

Site-directed and deletion mutagenesis by PCR was performed by designing DNA primers containing restriction enzyme sites for cloning and complementary sequence to *plr* except for the desired mutation or altered translational start site. Primer sequences are listed in Table 2-2. The plasmid pRL024 was used as the DNA template for PCR. Amplified fragments were subcloned into the vectors pTrc99C (Aman et al., 1988) or pMal-c2 (New England Biolabs, Beverly, MA) as indicated in Table 2-1. In some instances, due to difficulty in direct cloning, the PCR products were first ligated into the vector pT7Blue (Novagen, Inc.), and inserts of pT7Blue plasmid were then excised and subcloned into pTrc99C or pMal-c2. The vector pTrc99C contains the *lac*I gene, which represses the *trc* promoter unless induced with IPTG. A *Nco*I site (containing an ATG start site) at the 5' end of the multiple cloning site allowed for the expression of Plr mutants lacking additional vector-encoded amino acids. The multiple cloning site of pMal-c2 lies 3' to the *malE* gene resulting in expression of maltose binding protein (MBP)/Plr fusions when *plr* inserts were cloned in-frame with *malE*. The

wild-type signal sequence of the MBP is deleted in pMal-c2 to prevent secretion of expressed fusion proteins into the cell periplasm. The 5' and 3' end junctions of vector and insert were sequenced to verify the presence of the mutation(s).

A 726 bp internal deletion of *plr* was generated by utilizing restriction enzyme sites within the ORF. Digestion of pRL024 with *Bst*YI yields three DNA fragments. The 0.6 kb *Bst*YI fragment containing the 3' end of *plr* and downstream strain 64/14 DNA sequences was ligated to the 3.0 kb *Bst*YI DNA fragment of pRL024, which harbors putative promoter sequences of *plr*, the 5' end of *plr*, and pACYC184 DNA (including the tet^r gene and the origin of replication). The plasmid pSW029 contains the first 99 bp of *plr* ligated in-frame to the 3' end 180 bp of *plr*. The expressed protein, Plr-29, is a fusion protein consisting of the NH_2-terminal 33 amino acids and the C-terminal 60 amino acids of Plr.

Solublization of inclusion bodies and recombinant protein purification. When many of the plasmids containing *plr* mutations were transformed into *E. coli*, the expressed proteins formed insoluble inclusion bodies. To isolate the recombinant proteins, bacteria were first grown in media overnight, shaking at 37° C. Bacteria were lysed by passage through a French pressure cell and the inclusion bodies were concentrated by centrifuging the lysates at 8,000 X g for 10 min. Inclusion bodies were then isolated using a modification of a previously published method (Leong et al.,

1991). Pellets containing inclusion bodies were suspended in 1% vol/vol Triton-X 100 and rotated for 30 min at RT. Inclusion bodies were pelleted by centrifugation and washed two times with dH_2O. The inclusion bodies were initially treated with 6 M guanidine. Solublized proteins were then either dialyzed against 0.5 M guanidine or subjected to an initial dialysis against 1.5 M guanidine, and then followed by dialysis against 0.5 M guanidine. The precipitate was removed by centrifugation, and dialysis was continued against 150 mM KCl, 50 mM Tris-HCL, pH 8.0 followed by 50 mM NaCl, 50 mM Tris-HCL, pH 8.0. Dialyzed samples were filtered through membranes with a pore size of 0.2 µm, and then stored at -70° C. Recombinant proteins Plr-36, Plr-38, Plr-28, Plr-46, Plr-49, and Plr-62 were solublized by this method. The solublization of Plr-29 and Plr-61 was not successful using this protocol.

Maltose binding protein/Plr fusion proteins were soluble when expressed in *E. coli* and were purified by amylose-agarose affinity chromatography following the manufacturer's instructions (New England Biolabs). Soluble lysates of *E. coli* χ6060 (pMal-c2), *E. coli* χ6060 (pRL070), and *E. coli* χ6060 (pRL071) were applied to the affinity column and incubated at RT for one hour. Non-specifically bound proteins were removed by washing the column extensively with wash buffer consisting of 10 mM phosphate, 0.5 M NaCl, 1 mM sodium azide, 10 mM betamercaptoethanol, pH 7.0, followed by elution of bound proteins by the addition of 10 mM maltose to

the wash buffer. Factor Xa cleavage of MBP fusion proteins was also performed following the manufacturer's instructions (New England Biolabs).

The soluble Plr mutants Plr-38, Plr-45, and Plr-42 retained the NAD^+ binding activity demonstrated by Plr and were purified by NAD^+ affinity chromatography as described in Chapter 1.

Gel electrophoresis and Western blot assays. The labeling of proteins with [^{125}I]Na, the preparation of plasmin, SDS-PAGE, and Western blot analysis using anti-Plr polyclonal antibody for immunoblots and [^{125}I]plasmin for ligand blot assays, were performed as described in Chapter 1.

GAPDH activity assays of cell lysates. *E. coli* producing recombinant Plr proteins were grown overnight at 37° C as shaking cultures. Cultures were centrifuged and bacterial pellets washed two times with 50 mM KPO_4, pH 6.0 and suspended to 2 ml per gram of pellet with buffer. Cells were lysed by two passages through a French pressure cell. Unlysed bacteria were removed by centrifugation at 5,000 X g for 10 min at 4° C, and insoluble debris was cleared by centrifugation at 33,000 X g, 30 min, 4° C. Lysates were assayed for protein concentration and for GAPDH enzymatic activity following the same procedures outlined for determination of enzymatic activity for purified proteins in

the Materials and Methods of Chapter 1. Specific activities are expressed as μM NADH min^{-1} mg^{-1} protein extract.

Results

Analysis of the plasmin ligand blot. Standardized procedures were used to assess immunoreactivity and plasmin binding activity of recombinant proteins (see Materials and Methods of Chapter 1). Highly purified commercial streptokinase (Kabi), recombinant GAPDH of *Bacillus stearothermophilus*, and recombinant Plr were examined by immunoblot and plasmin ligand blot. The absence of anti-Plr polyclonal antibody reactivity for streptococcal proteins other than Plr was shown in figure 1-1 of Chapter 1. The lack of cross-reactivity of the antibody with the streptococcal plasminogen activator streptokinase was previously demonstrated (Broder et al., 1991). As depicted in figure 2-2, additional specificity of this antibody was demonstrated by its failure to react with *B. stearothermophilus* GAPDH, even though this protein has 50% identity with Plr at the predicted amino acid sequence level. The streptokinase bound both plasmin and glu-plasminogen. In contrast, Plr bound plasmin in this assay but failed to bind significant amounts of glu-plasminogen, indicating a higher affinity for plasmin compared to glu-plasminogen. Interestingly, strain 64/14 also has a much higher affinity for plasmin compared to glu-plasminogen (Broder et al.,

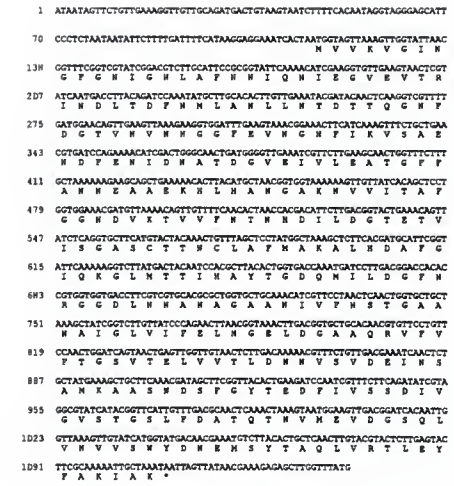


Figure 2-1. Nucleotide sequence of *plr*. The predicted amino acid sequence of Plr is indicated in single-letter code below the nucleotide sequence (Lottenberg et al., 1992a).

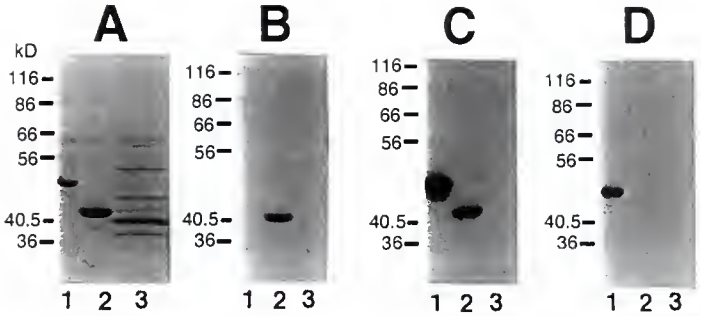
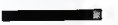
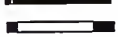


Figure 2-2. Western blot analysis of purified proteins for anti-sPlr antibody reactivity and plasmin binding activity. Proteins were electrophoresed on quadruplicate SDS-polyacrylamide gels. One gel was stained with Coomassie brilliant blue to visualize proteins (A), while the proteins on the other three gels were electrotransferred to nitrocellulose and probed with either anti-Plr antibody (B), [^{125}I]plasmin (C), or [^{125}I]glu-plasminogen (D). Lanes 1 contain streptokinase, lanes 2 contain Plr, lanes 3 contain recombinant *Bacillus stearothermophilus* GAPDH.

1989). The *B. stearothermophilus* GAPDH, an enzyme highly homologous to Plr, did not bind plasmin and indicated that specific regions are required for binding which are unique to Plr. Therefore, using this assay, binding interactions can be distinguished between either glu-plasminogen or plasmin with their respective ligands immobilized on nitrocellulose. The characterization of mutant Plr molecules generated in this study are summarized in Table 2-3.

In vitro analysis of recombinant Plr fusion mutations. To examine the contribution of C-terminal amino acids of Plr to its ability to bind plasmin, Plr-36, Plr-37, and Plr-38 were analyzed by ligand blot assay. Plr-37 was soluble when expressed in *E. coli* and was separated from irrelevant proteins using NAD^+ affinity chromatography. Plr-36 and Plr-38 formed insoluble inclusion bodies in *E. coli* and were solublized after isolation of the inclusion bodies prior to SDS-PAGE. All three C-terminal truncations of Plr reacted with the anti-Plr polyclonal antibody indicating that at least some of the antigenic epitope(s) of Plr lie within the NH_2-terminal 269 of the 335 native amino acids (figure 2-3B). When probed with ^{125}I-labeled plasmin, Plr-36, Plr-37, and Plr-38 demonstrated reduced binding activity compared to Plr, indicating that the C-terminus of Plr may play a role in plasmin binding (Figure 2-3C). Because Plr-37 has only the wild type C-terminal lysine deleted preceding the additional

Table 2-3. Summary of Plr mutations and the resulting phenotypes examined in this study.

Protein	Description	Product[a]	GAPDH Activity[b]	Soluble	Soluble Plasmin Binding[b]	Insoluble Plasmin Binding[b]
Plr	wild-type		+	+	+	N.A.
Plr	wild-type, denatured		-	+	+	N.A.
Plr-37	1 a.a. C-term. deletion		+	+	-	N.A.
Plr-38	38 a.a. C-term. deletion		-	-	-	-
Plr-36	76 a.a. C-term. deletion		-	-	-	-
Plr-28	4 a.a. C-term. deletion		-	-	-	-
Plr-45	C-term. substitution	 K→L	+	+	-	N.A.
Plr-46	C-term. substitution	 K→L	N.D.	-	+	N.D.
Plr-62	5 a.a. NH_2-term. deletion		N.D.	-	-	+
Plr-61	13 a.a. NH_2-term. deletion		N.D.	-	N.D.	+
Plr-49	33 a.a. NH_2-term. deletion		N.D.	-	-	N.D.
Plr-42	NH_2-term. substitution	 K→Q	N.D.	+	+	N.A.
Plr-29	242 a.a. internal deletion		N.D.	-	N.D.	+
Plr-70	MBP+Plr		-	+	-	N.A.
Plr-71	MBP+116 a.a. NH -term. deletion		-	+	-	N.A.
	MBP+Lac Z[a]		-	+	-	N.A.

[a] Plr amino acids, Deleted residues, substituted lysine, Non-Plr residues, Maltose binding protein, Lac Z[a]

[b] N.D., not done; N.A., not applicable

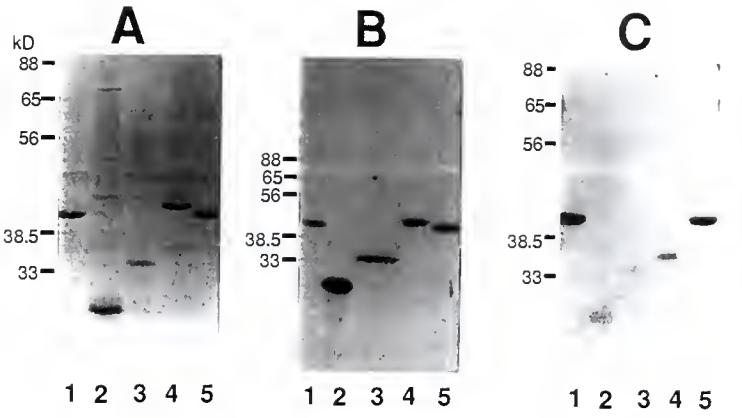


Figure 2-3. Western blot analysis of exonuclease III-generated *plr* mutations. Samples were electrophoresed on triplicate reducing SDS-10% polyacrylamide gels. One gel was stained with Coomassie brilliant blue to visualize proteins (A). Proteins resolved on the other two gels were transferred to nitrocellulose membranes, which were blocked and reacted with either anti-sPlr antibody (B), or with [^{125}I]plasmin (C) Lanes 1 and 5 contain Plr, lanes 2 contain Plr-36, lanes 3 contain Plr-38, and lanes 4 contain Plr-37.

vector-encoded amino acids, this residue may be important in mediating the binding of plasmin to Plr. None of the vector-derived sequences fused to the 3' end of *plr*-36, *plr*-37, and *plr*-38 coded for lysine residues and therefore the above results are consistent with a C-terminal lysine contributing to plasmin binding. The ~37 kDa plasmin binding protein contaminating the Plr-37 preparation may be *E. coli* GAPDH which bound to the NAD^+ column during Plr-37 purification.

To examine whether other regions of Plr in addition to the C-terminus were involved in plasmin binding, a 129 amino acid NH_2-terminal truncation of Plr was constructed. The full length *plr* ORF and the 5' end deletion DNA fragments were subcloned individually into the pMal-c2 vector creating in-frame fusions with the *malE* gene which encodes the MBP of *E. coli*. Expression of these gene fusions yielded the proteins Plr-70 and Plr-71, respectively. Purified fusion proteins and a MBP/$LacZ^{\alpha}$ fusion were examined for reactivity with anti-Plr antibody and plasmin binding activity (see figure 2-4). The proteins Plr-70 and Plr-71 are shown migrating close to their predicted ~M_r of 84,000 and 65,000, respectively, in figure 2-4A. The MBP has a ~M_r of 42,000 and the $LacZ^{\alpha}$ ~M_r 10,000, therefore the MBP/$LacZ^{\alpha}$ fusion migrates at ~M_r of 52,000. The anti-Plr antibody reacts with both Plr-70 and Plr-71 but not the MBP (figure 2-4B) indicating correct expression of the constructs and that

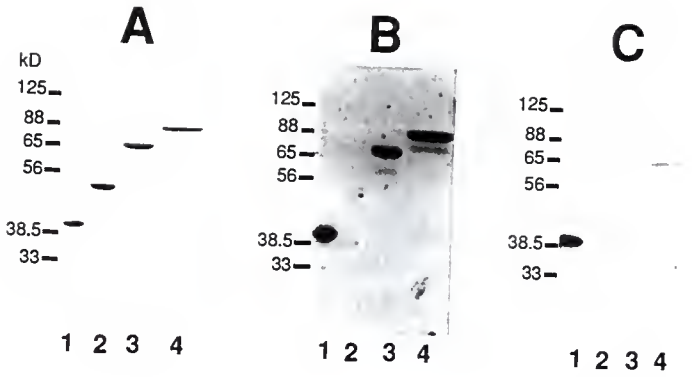


Figure 2-4. Western blot analysis of MBP/Plr fusion proteins. Samples were electrophoresed on triplicate reducing SDS-10% polyacrylamide gels. One gel was stained with Coomassie brilliant blue to visualize proteins (A). Proteins resolved on the other two gels were transferred to nitrocellulose membranes, which were blocked and reacted with either anti-sPlr antibody (B), or with [^{125}I]plasmin in buffer (C) Lanes 1 contain Plr, lanes 2 contain MBP/LacZ$^{\alpha}$, lanes 3 contain the MBP/NH_2-terminal Plr deletion fusion protein Plr-70 , and lanes 4 contain the MBP/Plr fusion protein Plr-71.

antigenic epitope(s) of Plr lie within the C-terminal 206 amino acids. The lower Mr reactive bands have been observed on previous blots, albeit with less intensity, and may represent partial degradation products of the fusion proteins. As shown in figure 2-4C, the two Plr fusion proteins as well as the MBP/LacZ$^{\alpha}$ fusion are deficient in plasmin binding relative to Plr. These results suggest that a NH_2-terminal region(s), in addition to a C-terminal region of Plr, is also required for wild-type levels of plasmin binding. NH_2-terminal residues may participate by direct interaction, or indirectly by contributing to conformational requirements necessary for optimal exposure of the C-terminal lysine. The removal of the MBP from the Plr-70 recombinant protein by Factor Xa would yield an additional eight amino acids fused to the native Plr NH_2-terminus. The Plr products from Factor Xa cleavage of Plr-70 and Plr-71 were identified by reactivity with anti-Plr antibody reactivity. Consistent with the analysis of the intact fusion proteins, neither Plr product bound [^{125}I]plasmin (data not shown). To further explore the contributions of both NH_2-terminal and C-terminal regions of Plr to the plasmin binding phenotype, it was necessary to construct non-fusion mutations of Plr.

In vitro analysis of non-fusion Plr mutations. To address whether the C-terminal and/or the penultimate lysyl residues of Plr were necessary for plasmin binding (see Plr sequence in figure 2-1), the plasmid pRL028, which has the

four 3' end codons of wild-type *plr* replaced with a termination codon, was constructed. Recombinant Plr-28 expressed in *E. coli* was purified from inclusion bodies and was solublized. However the preparation shown in figure 2-5 is an insoluble preparation. Plr-28 was compared to Plr for plasmin binding ability. The proteins are shown in figure 2-5A, and reactivity of Plr-28 with anti-Plr antibody was verified as shown in figure 2-5B. Similar to Plr-37, Plr-28 had reduced plasmin binding compared to Plr, indicating that the last four amino acids of Plr are necessary for wild type levels of plasmin binding (figure 2-5C). It therefore appeared likely that either or both of the C-terminal lysine residues were responsible for the plasmin binding activity of Plr.

To assess the contribution of each of the lysine residues, *plr* mutations were generated which substituted a leucine in place of one or the other lysines. The C-terminal lysine of Plr was substituted with a leucine in Plr-45, whereas in Plr-46 the penultimate lysine was replaced with a leucine and the C-terminal lysine was left intact. Plr-46 was purified and solublized as described for inclusion body proteins, whereas Plr-45 was soluble and was purified from an *E. coli* lysate by NAD^+ affinity chromatography as was performed for wild-type Plr (see Chapter 1). Additionally, to examine whether the NH_2-terminus contributed to plasmin binding as inferred from the MBP/Plr fusion proteins or alternatively if the terminal

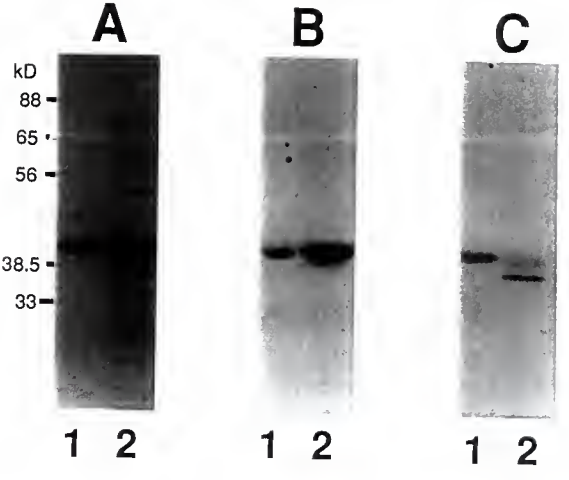


Figure 2-5. Western blot analysis of the C-terminal mutant Plr-28. Samples were electrophoresed on triplicate reducing SDS-10% polyacrylamide gels. One gel was stained with Coomassie brilliant blue to visualize proteins (A). Proteins resolved on the other two gels were transferred to nitrocellulose membranes, which were blocked and reacted with either anti-sPlr antibody (B), or with [^{125}I]plasmin (C). Lanes 1 contain Plr, and lanes 2 contain Plr-28. The plasmin binding protein migrating at a ~Mr slightly faster than Plr-28 is an *E. coli* contaminant protein.

lysine(s) was sufficient for wild type levels of binding, a 33 amino acid NH_2-terminal deletion of Plr, Plr-49, was constructed by PCR mutagenesis of *plr*. Plr-49 was purified and solublized from inclusion bodies. As a control to show that the solublization procedure had no effect on the ability of the mutant proteins to bind plasmin, wild-type Plr was also subjected to the solublization procedure. Plr, Plr-45, Plr-46, and Plr-49 reacted with anti-Plr antibody verifying correct expression of these Plr derivatives (figure 2-6B). When incubated with ^{125}I plasmin, Plr-46 appeared to bind approximately equivalent amounts of plasmin (figure 2-6C) as wild-type Plr, suggesting that the penultimate lysyl residue of Plr is not necessary for plasmin binding. However, the C-terminal lysyl residue is required for plasmin binding as evidenced by the observed reduction in plasmin binding by Plr-45. Additionally, EACA completely inhibited plasmin from binding to all of the proteins immobilized on the nitrocellulose membrane, consistent with the hypothesis that lysyl residues of Plr may interact with the lysine binding sites of plasmin (figure 2-5D). In agreement with the MBP/Plr fusion proteins, Plr-49 was also deficient in plasmin binding compared to Plr, suggesting that there are amino acids in addition to the C-terminal lysine which are necessary for wild type levels of binding.

If the above hypothesis were correct, then a Plr mutation which contains the NH_2-terminal portion which is

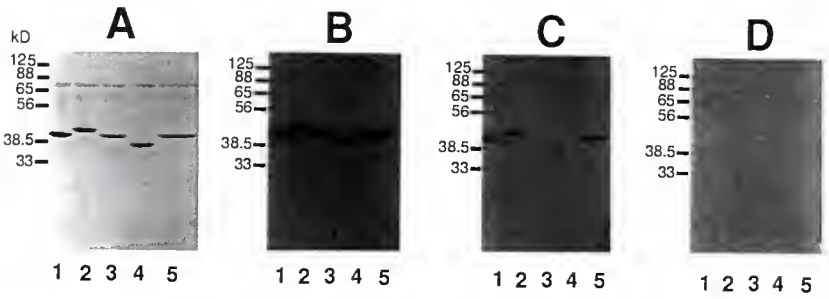


Figure 2-6. Western blot analysis of NH2-terminal and C-terminal mutations of Plr. Samples were electrophoresed on quadruplicate reducing SDS-10% polyacrylamide gels. One gel was stained with Coomassie brilliant blue to visualize proteins (A). Proteins resolved on the other three gels were transferred to nitrocellulose membranes, which were blocked and reacted with either anti-sPlr antibody (B), with [^{125}I]plasmin in buffer (C) or with [^{125}I]plasmin in buffer containing 20 mM EACA (D). Lanes 1 contain Plr, lanes 2 contain Plr-46 which has the penultimate lysine residue substituted with a leucine, lanes 3 contain Plr-45 which has the C-terminal lysine replaced with a leucine, lanes 4 contain the 33 amino acid NH_2-terminal deletion Plr-49, and lanes 5 contain Plr that has been subjected to the solublization protocol described in Material and Methods.

missing in Plr-49, fused to a C-terminal peptide of Plr should retain its ability to bind plasmin. To test this hypothesis, an in-frame internal deletion mutant of Plr, Plr-29, was constructed using available restriction enzyme sites in *plr*. Plr-29 contains the NH_2-terminal 33 amino acids fused to the C-terminal 60 amino acid of Plr. Plr-29 was expressed as inclusion bodies in *E. coli*, and was not able to be solublized prior to SDS-PAGE as was done with the other mutant Plr proteins. This protein migrates at a ~Mr of 10 kDa in the Coomassie brilliant blue stained gel of figure 2-7A and is recognized by anti-Plr antibody (figure 2-7B). A soluble preparation of Plr-28 is shown for comparison. Analysis of plasmin binding (figure 2-7C) revealed that Plr-29 does indeed bind plasmin. The plasmin binding ability of Plr-29 supports the concept that a NH_2-terminal region, in addition to the C-terminal lysine residue, is required for wild-type binding.

Interaction of the NH_2-terminus of Plr with plasmin may occur by direct binding of the ligand or possibly indirectly by positioning the C-terminal lysine in a suitable orientation to allow high levels of binding. To further explore NH_2-terminal contributions of Plr to plasmin binding, several additional mutations were made. Shorter 5' end deletions of *plr* were constructed and analyzed to more precisely delineate the contributing region(s) or residue(s). The mutant Plr-61 has the NH_2-terminal thirteen amino acids of native Plr

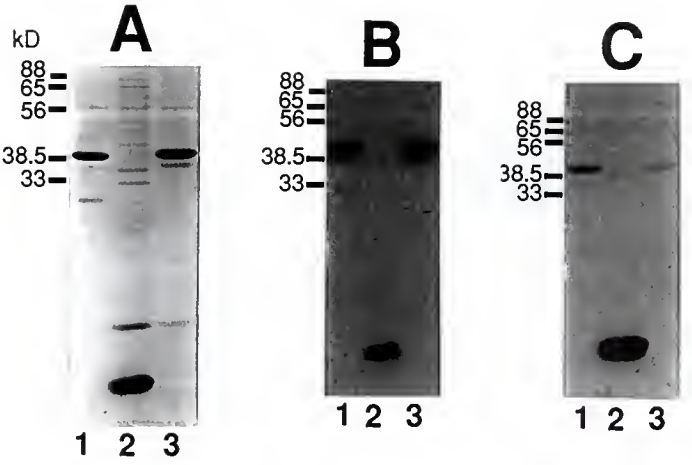


Figure 2-7. Anti-sPlr antibody reactivity and plasmin binding ability of the internal deletion Plr mutant, Plr-29. Samples were electrophoresed on triplicate reducing SDS-15% polyacrylamide gels. One gel was stained with Coomassie brilliant blue to visualize proteins (A). Proteins resolved on the other two gels were transferred to nitrocellulose membranes, blocked, and reacted with either anti-sPlr antibody (B), or probed with [^{125}I]plasmin. Lanes 1 contain Plr, lanes 2 contain Plr-29, and lanes 3 contain Plr-28.

deleted whereas Plr-62 has only the first five NH_2-terminal amino acids removed. Additionally, to test the possibility that the lysine^4 residue in close proximity to the NH_2-terminus of Plr could contribute to plasmin binding, Plr-42 was constructed which contains a glycine^4 substituted for the wild-type lysine^4. Plr-42 was expressed as a soluble protein and was purified by NAD^+ agarose affinity chromatography. Plr-61 and Plr-62 were in inclusion bodies but only Plr-62 was successfully solublized. Protein preparations were subjected to SDS-PAGE and stained with Coomassie brilliant blue to visualize the proteins as well as transferred to nitrocellulose to assay for plasmin binding activity (figure 2-8A). Both soluble and insoluble Plr-62 samples were assayed for plasmin binding activity. Plr-49, the 33 amino acid deletion of Plr which has reduced plasmin binding, is shown for comparison in figure 2-8B. Plr-42 retained wild-type levels of plasmin binding, which indicated that the lysine in close proximity to the NH_2-terminus does not participate in the binding activity as detected by this assay. Soluble Plr-62 has greatly reduced binding activity relative to Plr. This result would suggest that residues within the first five amino acids of recombinant Plr are required for plasmin binding in addition to the C-terminal lysine. In contrast, insoluble Plr-61, which is the larger of these two deletions, and insoluble Plr-62 have similar levels of plasmin binding as wild-type Plr. These

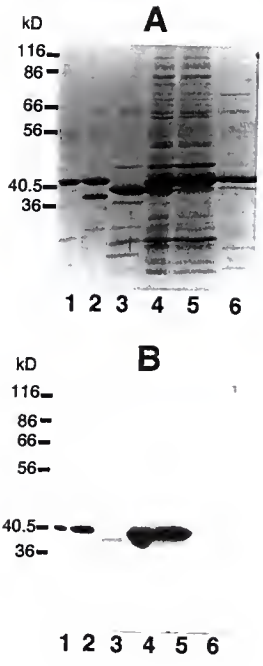


Figure 2-8. Plasmin binding ability of Plr mutants with NH_2-terminal alterations. Samples were electrophoresed on duplicate reducing SDS-15% polyacrylamide gels. One gel was stained with Coomassie brilliant blue to visualize proteins (A). Proteins resolved on the other gel was transferred to a nitrocellulose membrane which was blocked, and reacted with [^{125}I]plasmin (B). Lanes 1 contain Plr, lanes 2 contain Plr-42 which has the lys^4 substituted with gly^4, lanes 3 contain Plr-49 which is a 33 amino acid NH_2-terminal deletion of Plr, lanes 4 contain a crude insoluble preparation of Plr-61 which is a 13 amino acid NH_2-terminal deletion derivative of Plr, lanes 5 contain a similar preparation of Plr-62 which has only the first five amino acids of wild-type Plr removed, and lanes 6 contain soluble Plr-62.

differences in plasmin binding between soluble and insoluble preparations of NH_2-terminal Plr mutations have not been observed for soluble and insoluble preparations of C-terminal Plr mutants (e.g. Plr-28 shown in figures 2-5 and 2-7). These results raise the possibility that NH_2-terminal residues are required to position the C-terminal lysine in an accessible orientation for interaction with plasmin and that this position effect is lost in mutant Plr after being denatured with guanidine.

Assessment of GAPDH enzymatic activity of Plr mutations.

To generate a viable strain 64/14 containing a mutated Plr with reduced plasmin binding activity, GAPDH activity needs to be retained. Therefore it was necessary to assess the GAPDH activity of the mutant proteins. Lysates of *E. coli* χ6060 containing either the vector pACYC184, pRL024 harboring wild-type *plr*, or pRL037 containing a four base pair deletion of the 3' end of *plr*, were assayed for GAPDH activity in two separate experiments. The enzymatic activities of *E. coli* χ6060(pACYC184), *E. coli* χ6060(pRL024), and *E. coli* χ6060(pRL037) lysates were 11.7, 154.2, and 49.0 μM NADH min^{-1} mg $extract^{-1}$ respectively, in one experiment and 17.5, 185.5, and were 71.8 μM NADH min^{-1} mg $extract^{-1}$ in a second experiment. The average increase in specific activity of lysates containing Plr over host background alone was almost 12-fold, whereas the Plr-37 preparations had an approximately

4-fold increase over background activity. Additionally, purified Plr-45 which has the C-terminal lysine substituted with a leucine and demonstrated reduced plasmin binding similar to Plr-37, was glycolytically active as well, although the specific activity was reduced relative to the purified wild-type protein (data not shown). These experiments demonstrated that Plr-37 and Plr-45, each harboring a mutation of Plr that resulted in reduced plasmin binding ability, still retained glycolytic activity, albeit with an approximate minimum of 3-fold less activity than that of Plr, and therefore indicated it was possible to at least partially separate glycolytic enzyme activity from plasmin binding activity.

All of the 3' *plr* mutations which extended farther than the C-terminal lysine codon were expressed as insoluble proteins in *E. coli* hosts regardless of whether transcription occurred from the wild-type putative promoter contained within the *Bam*HI-*Hind*III streptococcal DNA fragment harboring *plr* or when PCR-generated mutations were subcloned behind and expressed from an inducible *trc* promoter. In contrast, wild-type *plr* and mutations of the C-terminal lysine codon were expressed as soluble, enzymatically active proteins.

Soluble cell lysates of *E. coli* χ6060(pRL036) and *E. coli* χ6060(pRL037), which express the exonuclease III generated C-terminal deletions of Plr, showed no increase in GAPDH enzymatic activity relative to *E. coli* χ6060(pACYC184), however the majority of mutant Plr visible by Coomassie

brilliant blue staining was visible in the insoluble fractions of the lysate preparations. Similarly, a soluble lysate of *E. coli* χ2602(pRL028), expressing the four amino acid deletion mutant Plr-28 protein, yielded an equivalent GAPDH activity of 10 μM NADH min^{-1} mg $extract^{-1}$ as *E. coli* χ2602(pTrc99C) harboring the vector alone. Insolubility of the recombinant proteins could account for the lack of enzymatic activity due to improper folding and/or an absence of the proteins of interest in the soluble fraction. Therefore conclusions cannot be drawn about the role of the deleted NH_2- and C-terminal regions other than the C-terminal lysine in the GAPDH activity of Plr.

Recombinant Plr was subjected to the solublization procedure and, importantly, this procedure did not affect the plasmin binding ability of Plr. However, the procedure did abolish GAPDH enzymatic activity of the protein, suggesting that Plr had not refolded properly. Therefore, definitive conclusions pertaining to the potential enzymatic activity of purified solublized mutant Plr proteins cannot be made.

The maltose binding protein fusion mutants, Plr-70 and Plr-71, were also assayed for enzymatic activity. There was no detectable activity for either protein, indicating the importance of the native NH_2-terminus for enzymatic activity as well as plasmin binding activity. The absence of activity may be due to improper folding of the putative nucleotide binding domain in Plr-71 and the lack of this domain in Plr-

70 resulting in deficiencies in NAD^+ binding, stable tetramer formation, or effects on the substrate binding domain.

A C-terminal lysine residue of Plr was determined to be essential for the plasmin binding activity of Plr in vitro. Analysis of additional mutant Plr molecules indicates that a C-terminal lysine was not sufficient for plasmin binding and that other regions were necessary as well. Mutant Plr molecules lacking or containing a leucine instead of the C-terminal lysine were soluble and retained GAPDH enzymatic activity. These studies have therefore generated gene candidates for substitution of *plr* in vivo to generate isogenic mutants of strain 64/14.

Discussion

The plasmin binding protein Plr has been hypothesized to be a plasmin receptor of group A streptococcal strain 64/14 (Broder et al., 1991). The *plr* gene was cloned previously from a strain 64/14 chromosomal DNA library (Lottenberg et al., 1992a). In this study, through genetic mutations of recombinant *plr*, specific amino acid residues of Plr have been examined for their contribution to plasmin binding activity. The goals of this series of experiments were to not only characterize the interactions between Plr and plasmin, but to also generate a non-plasmin binding mutant of Plr that retains glycolytic activity. A summary of the mutations generated in this study are presented in Table 2-3.

The amino acid lysine can efficiently elute plasmin from the surface of group A streptococci. Lysine and lysine analogs also prevent the association of plasmin with the bacterial surface (Broeseker et al., 1988). This inhibition of binding occurs in a concentration-dependent manner that suggests the involvement of the high-affinity lysine binding site (LBS) of plasmin. Furthermore, the domain of plasmin(ogen) facilitating the interaction is localized to the heavy chain which contains the LBS's (Broder et al., 1989). The deduced amino acid sequence of Plr reveals the presence of lysine residues in the C-terminus (see figure 2-1) that could potentially mediate the reversible binding of plasmin observed for intact streptococci and purified Plr (Lottenberg et al., 1992a). As demonstrated in figure 2-6, the presence of EACA, a lysine analog, inhibited the binding of plasmin to Plr, implicating the participation of either or both of these lysyl residues in this interaction. The results of analyses of C-terminal mutations of Plr supported this hypothesis. The C-terminal deletion fusion proteins Plr-36, Plr-37, and Plr-38 were all deficient in binding. Plr-37 has only the C-terminal lysine deleted, however the predicted vector encoded amino acids placed the penultimate lysine residue 17 amino acids proximal to its wild-type position and therefore prevented interpretation of its contribution to plasmin binding. Plr-28, a mutant protein with the four C-terminal wild-type amino acids deleted including both the penultimate and C-terminal lysine, was

also deficient in plasmin binding and so more definitively implicated the C-terminal lysines in the plasmin binding interaction. To assess the role of each of these lysine residues in plasmin binding, the single amino acid substitution mutants which contain a leucine residue in place of the lysine in either the C-terminal or the penultimate positions, Plr-45 and Plr-46, respectively, were constructed. The reduction of plasmin binding of Plr-45 compared to Plr verified that the C-terminal lysine of Plr was necessary for wild-type levels of binding. In contrast, there was no difference in plasmin bound by Plr-46 relative to Plr suggesting that the penultimate lysine residue may not participate in the binding interaction.

A penultimate lysyl residue, and in some cases arginine, is predicted from the DNA sequences to be present within the C-terminal four amino acids of GAPDHs from a wide spectrum of organisms including the non-plasmin binding GAPDH from *Bacillus stearothermophilus* (Branlant et al., 1989). A basic residue at this location could potentially contribute to the conformation of the protein and therefore not be available for interaction with plasmin. Consistent with this hypothesis, all C-terminal deletions of Plr extending beyond the penultimate lysine were expressed as insoluble proteins, as was Plr-46 containing a substitution of this residue. This is consistent with improper protein folding, although other factors resulting from high expression of foreign proteins in *E. coli* could also account for this effect.

Interactions of plasmin(ogen) with C-terminal lysyl residues have previously been reported for fibrinogen fragments, the physiological inhibitor alpha-2-antiplasmin, and for eukaryotic plasmin(ogen) receptors. By treating fragments of fibrinogen with carboxypeptidase B (CPB), which specifically cleaves C-terminal lysine and arginine residues, Christensen demonstrated that treated fragments would no longer bind to a plasminogen-Sepharose column (Christensen, 1985). Untreated fragments bound to the column and could be eluted by the addition of EACA, a lysine analog, thereby implicating C-terminal lysines of the fragments in the binding interaction.

The plasminogen activator urokinase, in solution or when bound to a specific eukaryotic urokinase receptor, can activate plasminogen to plasmin. However, enhanced activation occurs when plasminogen has undergone dramatic conformational change by binding either free lysine or a C-terminal lysine of a peptide through the high affinity lysine binding site (LBS) in kringle 1 (Violand et al., 1975). The study by Pannell et al., which addressed plasminogen activation by urokinase, revealed that pre-treatment of fibrin fragments with CPB reduced the rate of plasminogen activation by urokinase thereby supporting the role of a C-terminal lysine in plasmin(ogen) binding interactions (Pannell et al., 1988).

The major physiological inhibitor of plasmin, alpha-2-antiplasmin (AP), binds to plasmin by a two step mechanism

(Wiman et al., 1979). The first step can be inhibited by lysine, and it has been shown that the C-terminal lysine of AP interacts with the high affinity LBS of plasmin. The second step is a lysine-independent mechanism occurring at or near the active site of plasmin. Sasaki et al. demonstrated inhibition of the plasmin:AP complex with a peptide constituting the C-terminal 25 amino acids of AP (Sasakai et al., 1986). Additionally, they hypothesized that a penultimate lysine residue of AP may also contribute to the high affinity of AP for plasmin. This penultimate lysine lies seventeen amino acids from the C-terminus of AP. Sugiyama et al. demonstrated increased inhibition of the plasmin:AP complex formation using trypsin generated C-terminal peptides of AP that included this penultimate lysine residue (Sugiyama et al., 1988). Hortin et al. performed additional inhibition experiments using synthetic peptides containing arginine residues in place of the lysines to study the AP:plasmin interaction. Peptides containing either the C-terminal or penultimate lysine residue substituted with arginine resulted in a 9-fold and 5-fold reduction, respectively, in the inhibition of AP:plasmin complex formation compared to a peptide of the wild-type sequence (Hortin et al., 1989). Two hypotheses offered by these authors are that the internal lysines may be interacting with the low-affinity LBSs of plasmin, or that residues including the penultimate lysine contribute to a conformation of the

peptide which places the C-terminal lysine in a favorable position for binding to plasmin.

Recently an endothelial cell membrane protein, annexin II, was described that binds both tissue plasminogen activator (tPA) and plasminogen at different sites (Hajjar, 1991 and 1994). A C-terminal lysine residue of the receptor is thought to mediate the binding of plasminogen via the kringle domains. Hajjar showed a significant reduction of plasminogen binding to endothelial cells following CPB treatment whereas tPA binding was unaffected by this treatment (Hajjar, 1993).

In examining plasminogen binding to monocytoid U937 cells, Miles et al. used a series of synthesized peptides to inhibit glu-plasminogen from binding to the cell surface (Miles et al., 1991). The peptides contained lysyl residues at various positions in order to assess the importance of lysines in the binding interaction. They reported that peptides containing a C-terminal lysine residue were essentially as effective in the inhibition of plasminogen binding to the cell surface as were peptides containing both a C-terminal and an internal lysine. Peptides containing only an internal lysine would not inhibit plasminogen binding. Interestingly, peptides consisting of at least the terminal nineteen amino acids of alpha-2-antiplasmin, which includes its the penultimate lysine, were 4.5-fold more effective at competing plasminogen off the cell surface than other peptides which also contained a C-terminal lysine.

These data led the authors to speculate that perhaps structural features of proteins or peptides were important for plasminogen binding in addition to a C-terminal lysine residue. To further emphasize the requirement of a C-terminal lysine for surface plasminogen capture, U937 cells were treated with CPB, and this treatment resulted in a 67% loss of plasminogen binding relative to untreated cells. Treatment of the purified receptor molecule, the glycolytic enzyme alpha-enolase, with CPB resulted in a decrease in plasminogen binding activity using a ligand blot assay similar to the assay used in this study to characterize plasmin binding to Plr. Additionally, a synthetic peptide of the C-terminus of alpha-enolase (containing a C-terminal lysine) was able to inhibit plasminogen binding to untreated alpha-enolase (Miles et al., 1991).

Internal lysine residues have also been implicated in mediating plasminogen binding directly in lieu of C-terminal lysines. In fibrinolysis, tPA must be bound to fibrin to efficiently activate native glu-plasminogen, which is also fibrin bound. Glu-plasminogen is thought to be bound via a low affinity LBS interacting with internal lysine residues of intact fibrin in this tri-molecular complex (Nieuwenhuizen et al., 1983). Consistent with this low affinity alternative binding site, Pannell et al. demonstrated that removing C-terminal lysyl residues of intact fibrin by CPB had no effect on the ability of tPA to activate fibrin-bound plasminogen. Removal of C-terminal lysines was confirmed by amino acid

analysis of treated and untreated supernatants (Pannell et al., 1988).

The ligand blot assay used in these studies to assess plasmin(ogen) binding revealed that Plr binds plasmin with a greater avidity than glu-plasminogen. In contrast to streptokinase, Plr did not bind glu-plasminogen. This lack of binding suggested that the penultimate lysine of Plr may not interact with the low affinity LBS's of plasmin(ogen) as do penultimate lysine residues in proteins such as fibrin. It is possible that the penultimate lysine of Plr does play a minor role in plasmin binding but that the ligand blot assay is not sensitive enough to reflect such potential differences in binding affinity. However, the C-terminal lysine appears to be a major determinant.

Many group A streptococcal isolates, including strain 64/14, specifically bind plasmin or lys-plasminogen to a greater extent than for glu-plasminogen. Although there are streptococcal strains which bind both plasmin and glu-plasminogen, albeit with different affinities, these strains contain certain M protein serotypes which are reported to modulate the lower affinity binding to glu-plasminogen (Kuusela et al., 1992b). Furthermore, only lys-plasmin(ogen) and, to a lesser extent, isolated heavy chain of lys-plasmin containing the kringle regions, could efficiently compete bound plasmin from the cell surface of strain 64/14 which does not express a plasminogen binding M protein (Broder et al., 1991, M. D. P. Boyle, personal communication).

Specificity of ligand binding to the whole bacteria is further demonstrated by the inefficient binding of proteins which contain kringle regions homologous to those of plasmin(ogen), such as urokinase or tPA (DesJardin et al., 1989).

The reduced plasmin binding of NH_2-terminal deletion mutants of Plr, intact MBP-fusions with Plr as well as the resulting Factor Xa cleavage products indicated that the presence of a C-terminal lysine on a protein is not sufficient for plasmin binding activity. Therefore, in addition to a C-terminal lysine, NH_2-terminal residues may be required for wild-type levels of binding either by direct interaction or aiding in the optimal presentation of the C-terminal lysine.

There are plasmin(ogen) binding proteins which are reported to mediate binding through residues other than lysines. As noted above, Berge and Sjobring identified a 43-kDa plasmin(ogen) binding protein from M-protein type 53 group A streptococci (Berge and Sjobring, 1993). The molecule has homology to the M-like family of proteins which include IgG receptor proteins. These proteins have a conserved signal sequence for targeting to the cell membrane and a cell wall spanning region. M-like proteins are anchored to the cell membrane via a domain near the C-terminus thereby precluding the C-terminus in a plasmin binding role. However, plasminogen bound to the cell surface of these particular streptococci can be eluted by EACA, and

the region binding the plasminogen molecule to the cell surface harbors the high-affinity LBS. The in vitro analysis of the putative 43-kDa receptor was performed on acid extracted material from the bacteria, and therefore the receptor may be a cleavage product of the native molecule. Both the recombinant and the endogenous 43-kDa protein were treated with CPB prior to analysis of plasminogen binding, however the removal of any potential C-terminal lysines was not experimentally confirmed. Although the authors concluded that binding is not mediated via a C-terminal lysine, they have not yet demonstrated that inactivation of the gene encoding this protein results in a reduction of plasminogen binding to the bacteria.

Additional support for the contribution of a NH_2-terminal region(s) of Plr to plasmin binding was demonstrated by the reduced binding activity of Plr-49, a non-fusion 33 amino acid NH_2-terminal deletion of Plr. Furthermore, when this same NH_2-terminal region was fused to the C-terminal 60 amino acids of Plr, plasmin binding was restored.

To further delineate the contribution of the NH_2-terminus of Plr in plasmin binding, several additional mutations were generated. The mutant Plr-62, lacking the five NH_2-terminal amino acids, had substantial loss of plasmin binding activity when a purified soluble preparation of this protein was assayed. In recombinant Plr this region consists of {M} V V K V. The *B. stearothermophilus* GAPDH does not bind plasmin, and the C-terminus of this protein

does not contain a terminal lysine; instead a lysine is followed by a glycine and a terminal leucine. This GAPDH does, however, have an identical NH_2-terminus to that of Plr indicating that this sequence of residues is not sufficient for directly binding plasmin. Contributions from the NH_2-terminal methionine are unlikely since it is present in only 50% of the recombinant Plr and is not present on mature Plr isolated from group A strain 64/14 (Chapter 1). Additionally, Plr-42, which contains the lysine^4 substituted with glycine^4, bound wild-type levels of plasmin, thereby ruling out participation of this residue in the binding interaction. In addition to a C-terminal lysine, this would leave the three remaining valine residues of the NH_2-terminus as candidates for the necessary amino acids required for wild-type levels of binding.

The crude, insoluble preparations of Plr-62 and Plr-61, which is a thirteen amino acid NH_2-terminal truncation, both demonstrated equivalent amounts of plasmin binding relative to wild-type Plr. These results were in contrast to the lack of plasmin binding demonstrated by soluble Plr-62. However, the C-terminal deletion constructs containing wild-type NH_2-termini are deficient in binding regardless of whether the protein preparation has been solublized or not. Plr taken through the solublization procedure retains plasmin binding activity, although the protein does lose GAPDH enzymatic activity. Furthermore, the insoluble mutant Plr-46 retains its plasmin binding activity after solublization. These

results indicate that this procedure does not have any direct effects on plasmin binding ability. Even though proteins are denatured with SDS during SDS-PAGE, the SDS is removed following electrophoresis and limited refolding of the protein may occur during transfer of proteins from the polyacrylamide gels to nitrocellulose membranes. It is possible that NH_2-terminal residues are required for appropriate presentation of the C-terminal lysine for effective plasmin binding. When the NH_2-terminal region was removed, this positioning effect may have been lost. However in the crude preparations of insoluble NH_2-terminal Plr mutants, some of the molecules may have retained appropriate presentation of the lysine due to altered conformation of the protein in inclusion bodies. Appropriate presentation of the C-terminal lysine may be lost when these proteins are exposed to guanidine prior to SDS-PAGE, thus accounting for the differences in binding activity between the soluble and insoluble preparations of Plr-62.

A C-terminal lysine residue is necessary but not sufficient for the plasmin binding phenotype of Plr. The data suggest that additional residues are required to position the lysine for optimal accessibility to plasmin.

The soluble Plr mutants Plr-37 and Plr-45 which have reduced plasmin binding activity retained GAPDH enzymatic activity. Therefore, the genes encoding these proteins are candidates for replacement of wild-type *plr* in group A strain 64/14. Alternatively, an additional candidate is the gene

encoding the non-plasmin binding GAPDH from *Bacillus stearothermophilus*. Successful introduction of these alternate genes at the *plr* locus would enable the contribution of Plr to the plasmin binding phenotype of strain 64/14 to be assessed.

CHAPTER 3
GENERATION AND ANALYSIS OF ISOGENIC MUTANTS OF *plr* IN GROUP A STREPTOCOCCAL STRAIN 64/14

Group A streptococci are highly invasive organisms (Stevens, 1992). The mechanism(s) utilized by the bacteria to penetrate through tissue barriers has not yet been elucidated. It has been hypothesized that proteolytically active plasmin bound to the bacterial cell surface plays a role in this degradative process (Lottenberg et al., 1987). A useful approach to test this hypothesis is to compare the pathogenic potential of wild-type streptococci to mutant streptococci lacking the ability to bind plasmin.

Ideally, in studying putative virulence factors of bacteria, one would like to generate an isogenic strain in which the expression of only the factor of interest is eliminated. This strain can then be compared to the wild-type strain for virulence in an animal model. The initial goal of these studies was to insertionally inactivate *plr* in strain 64/14 in order to evaluate the role of the putative plasmin receptor, Plr, in the plasmin binding phenotype of group A streptococci. These isogenic strains could then be tested in a mouse model where organisms are inoculated into a subcutaneous air bleb (Raeder and Boyle, 1993). However, attempts at insertional inactivation of *plr* were

unsuccessful. The extensive homology of the predicted amino acid sequence of Plr with GAPDHs suggested that this may be a glycolytic enzyme (Lottenberg et al., 1992) and could therefore potentially be an essential gene of group A streptococci. Characterization of Plr revealed that it is a functional GAPDH enzyme. Furthermore, group A streptococci have only a single copy of *plr*. The failure of strain 64/14 to grow using carbon sources other than glucose was consistent with the inability to insertionally inactivate *plr* and suggested that *plr* was in fact an essential gene in group A streptococci.

Two alternative approaches were applied to generate isogenic derivatives of strain 64/14. One approach was a gene replacement strategy whereby the *gap* gene from *Bacillus stearothermophilus,* which encodes a GAPDH which does not bind plasmin, would replace *plr* on the streptococcal chromosome. In addition, in vitro analysis of mutant Plr proteins, as presented in Chapter 2, was performed to identify domains or residue(s) of Plr which were important for binding plasmin and to determine if these domains were distinct from those required for glycolytic function. These studies revealed that a C-terminal lysine of Plr was required for wild-type levels of plasmin binding, and some of these Plr mutations retained GAPDH activity. In the second approach presented in this Chapter, strategies were applied to successfully introduce these mutations at the *plr* locus in strain 64/14. The strains expressing mutated Plr molecules were compared to

wild-type strain 64/14 in vitro for the ability to capture plasmin on the bacterial surface. Additional experiments are presented herein which further examined plasmin binding components of strain 64/14 and the cellular localization of Plr.

Materials and Methods

DNA manipulations and plasmids. DNA manipulations used in construction of plasmids was described in the Materials and Methods of Chapter 2. DNA hybridization studies were performed as outlined in the Materials and Methods of Chapter 1. The plasmids used in these studies are summarized in Table 3-1, and the construction of these plasmids is described in detail below.

Construction of integration plasmids. Cointegrative plasmids were constructed using the plasmids pRL024, which harbors wild-type *plr*, and pRL037, a derivative of pRL024 containing a 3' end deletion of *plr* generated by exonuclease III. The plasmids pRL024 and pRL037 were subjected to identical DNA manipulations as shown schematically in figure 3-1. The first 660 bp of *plr* and all of the strain 64/14 upstream sequences were removed by digesting the plasmids with *Bst*EII and *Hind*III. The DNA overhangs were blunt ended using Klenow fragment and dNTPs, and the linear DNA was ligated. These plasmids were linearized by digesting at a

TABLE 3-1. Plasmids constructed for group A strain 64/14 mutagenesis studies.

Plasmid	Plasmid Description	Marker[a]
pSW024	The 5' one third of *plr* and all of the strain 64/14 upstream sequences contained on the 2.2 kb insert of pRL024 were removed. The plasmid was then digested with *Sal*I and the overhangs blunt ended. The Ω Km^r cassette was ligated downstream of *plr* in a vector encoded *Sal* I site.	cm kan
pSW037	The plasmid pRL037, containing an exonuclease III generated 3' end deletion of *plr* (see Chapter 2), was subjected to the identical DNA manipulations as described for the generation of pSW024.	cm kan
pSW025	The 2.7 kb insert of pRL015 harboring *plr* was subcloned into a 2.2 kb fragment of pACYC184. The putative promoter of pSW025 transcribes in the opposite direction to the tet^r promoter.	tet
pSW026	This plasmid is an inverse PCR derivative of pSW025 that has *Nco*I and *Sma*I sites substituted in place of the precisely deleted *plr* ORF. The plasmid pSW025 was amplified by PCR using the inverse primers. The amplified product was digested with *Sma*I and ligated to itself to generate pSW026.	tet
pSW027	pSW026 was partially digested with *Bam*HI, and the protruding DNA overhangs were blunt ended. The overhangs of *Bam*HI digested Ω cassette were also blunt ended. The Ω cassette fragment was then ligated with linearized pSW025 to yield pSW027.	tet kan
pSW053	The *gap* ORF of *B. stearothermophilus* was amplified by PCR, and the 1 kb amplified DNA fragment digested with *Nco*I and *Sma*I. This insert was ligated into *Nco*I/*Sma*I digested pSW027 to generate pSW053.	tet kan

TABLE 3-1.--Continued.

Plasmid	Plasmid Description	Marker[a]
pSW063	The *plr-63* mutation, which has two additional codons, a glycine (GGT) and a leucine (CTT) codon added to the 3' end of the wild-type *plr* was cloned into the *Nco*I and *Sma*I sites of pSW027.	tet kan
pSW045	The *plr-45* ORF, which has the 3' end codon for lysine substituted with a leucine codon, was excised from pRL045. pRL045 was digested with *Bam*HI and the DNA overhangs blunt ended. The plasmid was then digested with *Nco*I and the resulting *plr-45* 1 kb insert was ligated into *Nco*I/*Sma*I digested pSW027 to yield pSW045.	tet kan

[a]cm, chloramphenicol; kan, kanamycin; tet, tetracycline

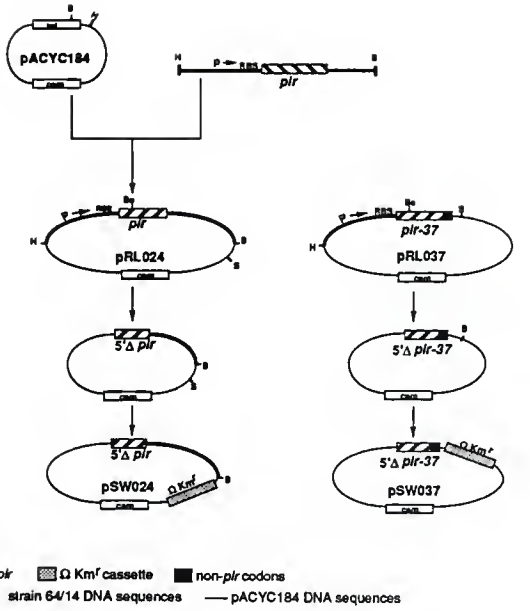


Figure 3-1. Construction of cointegrative plasmids for *plr* mutagenesis of strain 64/14. The plasmids pRL024 and pRL037 described in Chapters 1 and 2, respectively, were subjected to identical manipulations. Plasmids were digested with *Hind*III and *Bst*EII to remove the 5' end one third of the *plr* ORF and upstream streptococcal sequences. The Ω cassette, encoding a kanamycin resistance gene was ligated into the unique *Sal* I site of these plasmids to yield pSW024 and pSW037 containing wild-type 3' end *plr* and a 3' end mutation of *plr*, respectively. See text for further details. Abbreviations: H, HindIII; S, *Sal*I; Bs, *Bst*EII; E, *Eco*RI; B, *Bam*HI; P, putative promoter elements; RBS, ribosomal binding site; Km^r, kanamycin resistance gene; cam, chloramphenicol resistance gene; tet, tetracycline resistance gene. The solid box of *plr-37* represents predicted vector encoded sequences.

*Sal*I site downstream of the *plr* insert, and the overhangs blunt ended. A 2.3 kb *Sma*I fragment consisting of the Ω Km^r cassette was ligated downstream of *plr* into the blunt ended *Sal*I site to generate pSW024. The Km^r gene of the Ω cassette is flanked by transcriptional and translational terminators. Additionally, the gene product is functional in both *E. coli* and streptococcal hosts (Fellay et al., 1987).

Construction of the *plr* gene replacement vector. The plasmid pSW027 was designed to allow replacement of *plr* on the streptococcal chromosome with either alternative *gap* genes or *plr* mutations. Plasmid construction is shown schematically in figure 3-2. The 2.7 kb insert of pRL015 (Lottenberg et al., 1992) harboring *plr* was excised by *Eco*RI digestion and blunt ended with Klenow fragment and dNTPs. This insert was subcloned into a 2.2 kb fragment of the vector pACYC184 which had been digested with *Ava*I and blunt ended, and then digested with *Xmn*I to generate pSW025. This plasmid retains the tetracycline resistance (tet^r) gene of pACYC184 and the origin of replication. The promoter of *plr* transcribes in the opposite direction to the tet^r gene promoter.

The *plr* ORF of pSW025 was precisely deleted and replaced with *Nco*I and *Sma*I restriction enzyme cloning sites by performing inverse PCR on pSW025. The forward primer

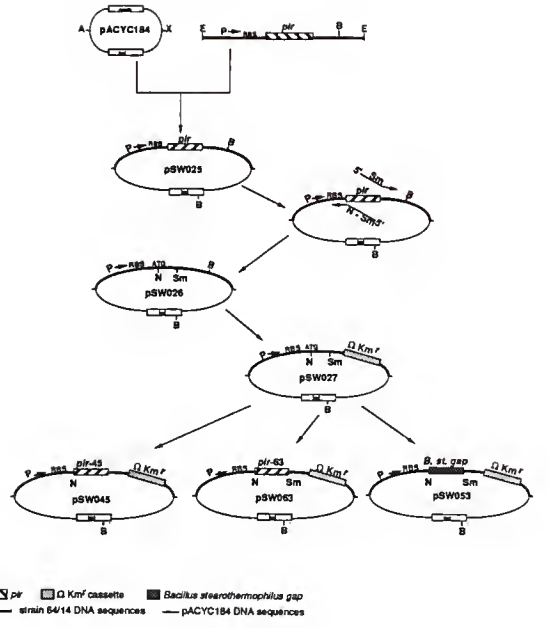


Figure 3-2. Plasmid construction for the replacement of wild-type *plr* with *gap* from *Bacillus stearothermophilus* or with *plr* mutations in strain 64/14. The insert of pRL015 containing *plr* and streptococcal flanking DNA sequences was subcloned into pACYC184 to yield pSW025. Inverse PCR was used to delete the *plr* ORF leaving flanking regions intact in pSW026. Partial *Bam*HI digestion of pSW026 allowed ligation of the Ω cassette within the streptococcal homologous sequences forming pSW027. The ORFs *plr*-45, *plr*-63, and *plr*-53 were subcloned into pSW027 to yield pSW045, pSW063, and pSW053, respectively. See text for further details. Abbreviations: E, *Eco*RI; B, *Bam*HI; N, *Nco*I; Sm, *Sma*I; X, *Xmn*I; A, *Ava*I; P, putative promoter elements; RBS, ribosomal binding site; Km^r, kanamycin resistance gene; cam, chloramphenicol resistance gene; tet, tetracycline resistance gene.

contains a *Sma*I site followed by wild-type strain 64/14 sequences that are 3' to the *plr* stop codon. The DNA sequence of the forward primer was 5'-CGCCCGGGTA TCCATGGATGATTTCCTCCTTATGAAAAT-3'. The reverse primer contained complementary sequence 5' to *plr* followed by an *Nco*I site at the *plr* ATG translational start site and then a *Sma*I site. The DNA sequence of the reverse primer was 5'-CGCCCGGGTTAGTTATAACGAAAGAGAGCT-3'. The plasmid pSW025 was amplified by PCR using the inverse primers. The amplified product was digested with *Sma*I and ligated to generate pSW026. The plasmid pSW026 was partially digested with *Bam*HI, and the protruding DNA overhangs were blunt ended. The 2.3 kb Ω Km^r cassette was excised from pSW024 by *Bam*HI digestion, and the overhangs were blunt ended. The Ω cassette fragment was then ligated with the linearized pSW026 to yield pSW027.

The *gap* ORF of *B. stearothermophilus* was amplified by PCR using the primers, RL54 5'-GCCCATGGCAGTCAAAGTGGGAATC-3' and RL53 5'-CGCCCGGGTTACAGCCCTTTCGAGGCG-3', designed by using the published DNA sequence (Branlant et al., 1983) and the plasmid pBS*gap* (kindly provided by Dr. C. Branlant) as template. The forward and reverse primers contained a *Nco*I site and a *Sma*I site, respectively, for subsequent cloning into pSW027.

The *plr*-63 mutation, containing a glycine and a leucine codon following the wild-type *plr* 3' end terminal lysine

codon, was amplified by PCR using RL22 (see Table 2-2) and RL63 5'-CGCCCGGGTTAAAGACCTTTAGCAATTTTTGCGAAGT-3' as oligonucleotide primers, and the plasmid pSW025 as DNA template. *Nco*I and *Sma*I digested *B. stearothermophilus gap* and *plr*-63 inserts were cloned into the *Nco*I and *Sma*I sites of pSW027 to generate the plasmids pSW053 and pSW063, respectively. The *plr*-45 insert was prepared by digesting pRL045 with *Bam*HI, blunt ending the overhangs, and then digesting with *Nco*I. This insert was cloned into *Nco*I and *Sma*I digested pSW027 to yield the plasmid pSW045. These plasmids can be linearized at the unique *Bam* HI site on the vector. When introduced into strain 64/14, a double cross-over event would potentially occur between homologous regions resulting in integration of the DNA into the chromosome.

Electroporation of DNA into strain 64/14. The plasmids pSW024, pSW037, and pSW053 were introduced into strain 64/14 as circular DNA using electroporation. The plasmids pSW053, pSW045, and pSW063 were introduced as linear DNA by digesting the plasmids at the unique *Bam*HI site prior to electroporation. DNA was electroporated into strain 64/14 following the method of Simon and Ferretti (Simon and Ferretti, 1991) or a similar protocol by M. Caparon (personal communication). A 5 ml starter culture of Todd Hewitt broth with 0.3% yeast extract (THY) and 20 mM glycine was grown as a standing culture, 37° C, overnight. The culture was then diluted with the same medium so that the initial optical

density (O.D.) was 0.06 to 0.08 when measured at an absorbance of 600_{nm}. The culture was incubated for 1 to 2 hrs at 37° C until the O.D. $_{600nm}$ was approximately 0.20. Bacteria were pelleted by centrifugation at 14° C, suspended in 5 ml of the spent culture medium, and then subjected to heat shock for 9 min at 43° C. Cells were washed two times in 15% glycerol and suspended in a final volume of 1 ml with 15% glycerol. Between one and five micrograms of DNA and 100 µl of cells were added to pre-chilled electroporation cuvettes, 0.5 cm electrode gap width (Biorad). The cells were subjected to a single pulse of 1.75 kV, 400 ohms. The cuvettes were subsequently kept on ice for 45 min, the bacterial suspension was diluted in 10 ml fresh THY broth, and then incubated at 37° C for 1 hr. Bacteria were pelleted by centrifugation, suspended to 0.5 ml THY broth and plated on THY agar containing 500 µg/ml kanamycin.

Bacterial strains and growth conditions. Bacterial strains used in these studies are described in Table 3-1. *E. coli* χ2602 harboring plasmids were grown overnight as shaking cultures at 37° C in Luria both. Tetracycline was added to the media at a concentration of 34 µg/ml, and kanamycin at 10 µg/ml where appropriate. Streptococcal strains were grown overnight as standing cultures in Todd Hewitt broth containing 0.3% yeast extract (THY) at 37° C. Strains containing the Km^r gene were grown in the presence of 300 µg/ml kanamycin in THY broth or 500 µg/ml on THY agar plates.

TABLE 3-2. Derivatives of streptococcal group A strain 64/14.

Strain	Description
64/14-24	Circular pSW024 DNA was integrated into strain 64/14 via a single crossover event within the region of homology extending from the 3' two thirds of *plr* to the *Bam*HI site downstream. The Ω Km^r cassette is located 3' to *plr* within the integrated pACYC184 sequences.
64/14k	A derivative harboring the Ω Km^r cassette on the chromosome approximately 400 bp downstream of *plr* at the former *Bam*HI site. The cassette was introduced by electroporation of linear DNA, and there are no integrated vector sequences.
64/14k-53	Circular pSW053 was introduced into strain 64/14 by electroporation. The plasmid integrated into the chromosome via a single crossover event immediately 5' to the *plr* gene. In strain 64/14k-53, the *B. stearothermophilus gap* gene is followed by the Ω Km^r cassette and pACYC184 plasmid sequences which are located 5' to the *plr* ORF. Both Plr and *B. stearothermophilus* GAPDH are expressed in strain 64/14k-53.
64/14k-45	The *plr* gene has been replaced by *plr-45*, which has the terminal lysine codon replaced with a leucine codon. The Ω Km^r cassette is located approximately 400 bp downstream of *plr* at the former *Bam*HI site.
64/14k-63	The *plr* gene has been replaced by *plr-63*, which has an additional glycine codon and a leucine codon immediately 3' to the wild-type terminal lysine codon. The Ω Km^r cassette is located in the same position as in strain 64/14k and strain 64/14k-45.

Serial passage of strain 64/14k-53 was performed without kanamycin selection to allow a potential second crossover event of the cointegrated plasmid to occur. One microliter of an overnight culture of strain 64/14k-53 was used to inoculate 100 ml of THY and grown as a standing overnight culture at 37° C. One microliter of this overnight culture was then used to inoculate 100 ml of fresh THY. This process was repeated six additional times. Bacteria were then plated on THY agar without selection. To screen for excision of the Ω Km^r cassette, two hundred colonies were patch plated on THY agar plates with and without antibiotic selection.

Protein purification and characterization. Purification of soluble proteins by NAD^+ affinity chromatography, Western immunoblot and ligand blot, generation of plasmin, and iodination of proteins were performed as described in the Materials and Methods of Chapters 1. The GAPDH activity assay of bacterial lysates is described in the Materials and Methods of Chapters 2.

Plasmin binding assays using whole bacteria. Bacteria were grown as standing overnight cultures at 37° C. Kanamycin selection of bacteria was applied as indicated in the results and figures. Cultures were pelleted by centrifugation, washed three times with PBS, and suspended to 20% wet pellet weight/volume with Veronal buffered saline, which contains 0.28 M NaCl and 10 mM sodium diethyl

barbiturate, pH 7.35, with 0.25% gelatin (VBS-gel). For [^{125}I]plasmin binding experiments, approximately 50,000 cpm of plasmin (with a minimum specific activity of 8 x 10^{11} cpm mg^{-1}) in a 100 µl volume was added to 100µl of the cell suspensions in borosilicate test tubes. Samples were mixed and incubated at 37° C for one hour. Two milliliters of VBS-gel was added to each tube, and the cells then were pelleted by centrifugation at 2,000 x g for 5 min, the supernatant aspirated, and the pellet suspended with 2 ml of VBS-gel. This washing procedure was repeated two more times before assaying the bacterial pellets in a Beckmann gamma 4000 model gamma counter. Background counts were determined by adding 100 µl of buffer without bacteria. The data are presented as the mean percentage of counts bound. The percentages were calculated by subtracting the background cpm of ligand alone from cpm bound by the bacteria divided by the total cpm of ligand offered multiplied by 100. Isolates were assayed in duplicate or triplicate as indicated in the tables.

For assays using unlabeled plasmin, cells were prepared exactly as described for the radiolabeled plasmin assays. Two hundred microliters of VBS-gel containing 25 µg/ml plasmin (~0.6 µM) was added to 200 µl of bacteria and incubated at 37° C for 1 hr. Cells were washed as described above. Four hundred microliters of VBS-gel containing 450 µM S-2251 was used to suspend washed pellets. S-2251 is D-Val-Leu-Lys-paranitroaniline, which is cleaved by plasmin on the carboxyl side of the lysine and results in the release of

paranitroanilide. Samples were incubated for 1 hr at 37° C before the reaction was terminated by the addition of 400 µl of 10% vol/vol acetic acid. Bacteria were pelleted by centrifugation, and the absorbances of the supernatants were measured with a spectrophotometer at an absorbance of 405 nm. Background activity was determined by adding 100 µl of buffer without bacteria. Plasmin activity is presented as the mean of the absorbances minus the background activity.

Assays were also performed to measure the capture of surface plasmin activity by bacteria when grown in the presence of plasma. One hundred µl of an overnight culture were used to inoculated 2 ml of THY containing 30% human plasma in borosilicate test tubes. Control samples consisted of THY media without plasma inoculated with strain 64/14 or THY with plasma and no bacteria to measure non-specific background activity. All tubes were incubated for 6 hr at 37° C. Bacteria were washed three times and then normalized for growth rate by suspended pellets to equivalent O.D. 600_{nm}. The streptococci were then incubated in 400 µl VBS-gel containing 450 µM S-2251 for up to 2 hr and the enzymatic reaction terminated as described for the assay above. Data are presented as the mean of the absorbances minus the background activity.

Protease treatment of streptococci. Bacteria were treated with either trypsin or carboxypeptidase B and then compared with untreated strain 64/14 for plasmin binding

activity. Two liters of THY were inoculated with strain 64/14, and bacteria were grown as a standing culture overnight at 37° C. Bacteria were pelleted by centrifugation and washed with PBS, and suspended to 25 ml with PBS. Chloramphenicol was added to a final concentration of 120 µg/ml, and cells were incubated for 30 min at RT. Hyaluronidase was added at 1800 U/ml (Sigma), and incubation was carried out for 30 min at 37° C to remove the bacterial capsule, ensuring that exposure of surface proteins could potentially be maximized. Cells were then washed two times in 0.1 NaCl, 0.025 Tris-HCL, pH 8.0 and suspended in a final volume of 5 ml using the same buffer. Seven hundred microliters of cell suspension were added to each microcentrifuge tube. For trypsin samples, 70 µl of a 100,000 U/ml trypsin stock solution (Calbiochem, LaJolla, CA) were added to the tubes, and incubations carried out at 37° C. Trypsin was inhibited by the addition of 12 µl of 25 mg/ml aprotinin (Sigma). For carboxypeptidase B treated samples, bacteria were pelleted by centrifugation, suspended in 300 µl of 0.5 M NaCl, 1 mM $ZnCl_2$, 0.05 M Tris-HCL pH 7.6 containing 100 U/ml carboxypeptidase B (Calbiochem), and then incubated at 37° C. The CPB was inhibited by the addition of 12 µl of 100 mM benzylsuccinic acid (Sigma). Bacterial samples containing protease and the inhibitor, or inhibitor alone were added as controls for efficiency of the inhibitor, and to examine the effect of the reagents in the subsequent plasmin binding assay. Samples were incubated for the

indicated time points, pelleted by centrifugation, and washed three times with VBS-gel. Pellets were suspended to 20% wet pellet weight/volume with VBS-gel, and the [^{125}I]plasmin binding assays were performed as described above. All experiments were performed at least two times.

Iodination of streptococci. Whole bacteria were reacted with free [^{125}I]Na to label proteins exposed on the surface. Bacteria were grown and prepared exactly as described above for protease treatment of streptococci. The following was added to 5 ml of the cell suspension: 670 µl of H_2O_2 (using a 1:1000 dilution of a 30% solution), 250 µl of lactoperoxidase (2.5 mg/ml stock), and 3 mCi of [^{125}I]Na (Amersham Life Science). The reaction was allowed to proceed for 10 min at RT before being terminated by the addition of 30 ml PBS containing 0.04% sodium azide. Bacteria were washed three times as described above, and suspended in 5 ml PBS. Bacteria were treated with mutanolysin as described in Chapter 1. Both the mutanolysin extract and the remaining protoplasts were subject to electrophoresis on duplicate polyacrylamide gels. One gel was stained with Coomassie brilliant blue to visualize the protein profiles and the other gel was dried on Whatman filter paper and exposed to autoradiography film at -70° C.

Phosphoglycerate Kinase Assay (PGK). PGK enzymatic activity was measured based on a previously published

protocol (Maitra and Lobo, 1971). Various dilutions of either mutanolysin extract or soluble cytoplasmic streptococcal lysates were added to 900 µl of the reaction buffer (50 mM triethanolamine, 10 mM $MgCl_2$, 45 µM NADH, pH 7.4), 20 µl 0.1 M 3-phosphoglyceric acid, 20 µl 0.1 M ATP, 50 µl 0.1 M cysteine HCl, and 4 µl of a 0.6 mg/ml solution of purified Plr. Controls consisted of performing the assays without the addition of ATP or without the addition of bacterial extracts to the reaction mixture. There was never any spontaneous conversion of NADH to NAD^+ without the addition of ATP and bacterial extract to the reaction mixtures. The oxidation of NADH to NAD^+ was monitored spectrophotometrically at an absorbance of 340_{nm} at 20 sec intervals over a 4 min time period using a Beckman model DU-70 spectrophotometer (Beckman Instruments, Inc, Fullerton, CA). The molar absorption coefficient of 18×10^{-3} (Segel, 1976) was used to express specific activities as µM NAD^+ min^{-1} mg protein $extract^{-1}$.

Results

The use of integration plasmids to introduce mutations of *plr* into strain 64/14. The *plr* gene could not be insertionally inactivated and appeared to be an essential gene in group A streptococci. Therefore, an approach to generate an isogenic mutant of *plr* in strain 64/14 to address the role of Plr as a plasmin receptor was to introduce a

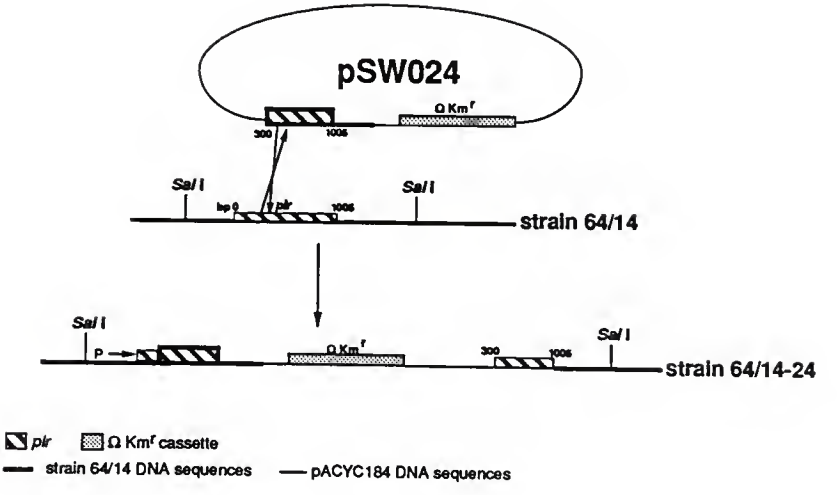


Figure 3-3. Integration of plasmid pSW024 into the strain 64/14 chromosome. pSW024 was electroporated into strain 64/14 and a single crossover via homologous recombination event resulted in strain 64/14-024. See text for details.

mutation in *plr* that would result in expression of a glycolytically active protein with reduced plasmin binding ability. The integrative plasmid pSW037 is a derivative of pRL037 that expresses Plr-37. This soluble recombinant protein is a C-terminal mutation of Plr that remains glycolytically active in an *E. coli* host and has reduced plasmin binding activity relative to wild-type Plr. The plasmid pSW024 is a derivative of pRL024 that encodes wild-type *plr* and was used in these experiments as a positive control. The replicon of these plasmids is not functional in the streptococcal host and therefore integration into the chromosome must occur for continuous expression of the Km^r gene located on the plasmid. Homologous recombination via a single crossover event should occur within the 3' end regions of *plr* or *plr-37* on the plasmids with *plr* on the chromosome (see figure 3-3). Bacteria transformed with circular pSW024 repeatedly yielded Km^r colonies, generating strain 64/14-24, whereas bacteria transformed with circular pSW037 did not yield any viable colonies.

DNA hybridization analysis was performed for strain 64/14-24 isolates. Either a *plr* ORF probe or an Ω cassette probe was reacted with digested chromosomal DNA extracted from several of the strain 64/14-24 Km^r transformants (figure 3-4). A *Sal*I digest of strain 64/14 chromosomal DNA yielded a 3.3 kb hybridizing fragment when probed with *plr* (consistent with the results of DNA hybridization studies of strain 64/14 in Chapter 1), whereas the strain 64/14-24

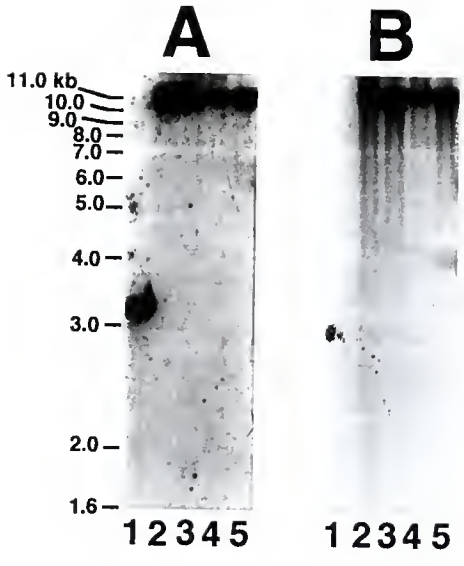
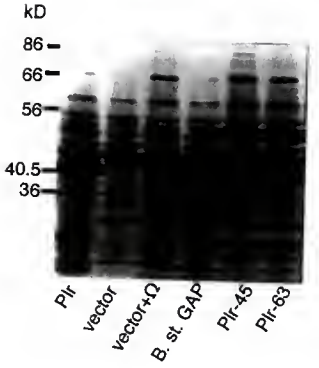


Figure 3-4. DNA hybridization analysis of chromosomal DNA from strain 64/14-24 transformants. DNA was digested with *Sal*I, electrophoresed on duplicate 0.7% agarose gels, and transferred to nylon membranes. The membranes were then reacted with a [^{32}P]dCTP-labeled probe consisting of either the *plr* ORF (A), or the 2.3 kb Ω cassette (B). Membranes were hybridized and washed at room temperature, and subjected to autoradiography. Lanes 1 contain strain 64/14 chromosomal DNA, and lanes 2,3,4 and 5 contains chromosomal DNA from individual strain 64/14-24 transformants.

isolates yielded a 10 kb fragment. This was the expected size for the *Sal* I fragment after the integration of the 6.7 kb plasmid. Additionally, hybridization with the Ω cassette probe also yielded a 10 kb-reactive fragment which was absent in the wild-type DNA. The 10 kb band was consistent with the size predicted for integration of the cassette followed by pACYC184 sequences. Therefore, these analyses confirmed the integration of pSW024 into the streptococcal chromosome at the *plr* locus. The generation of strain 64/14-24 indicated that it was possible to introduce DNA constructs into strain 64/14 at the targeted location and to integrate a selectable marker downstream of *plr* on the chromosome.

Gene replacement strategy to introduce mutations of *plr* into strain 64/14.

Genes encoding the *B.stearothermophilus gap* or *plr* mutations are expressed when cloned into the vector pSW027 and possess GAPDH activity in *E. coli*. The recombinant genes subcloned into pSW027 used in the gene replacement strategy must express products that possess GAPDH enzymatic activity. Recombinant proteins are highly expressed from the streptococcal *plr* promoter of pSW027 in *E. coli* harboring the plasmids of interest as shown on the Coomassie brilliant blue stained polyacrylamide gel of figure 3-5. *E. coli* χ2602 (pSW025), *E. coli* χ2602 (pSW045), *E. coli* χ2602 (pSW063), and *E. coli* χ2602 (pSW053) yielded GAPDH enzymatic activities of



Sample	GAPDH activity (μM NADH min^{-1} mg extract^{-1})	Fold ↑
vector	1.7	-
vector+Ω	1.7	-
Plr	100.5	60.2
B. st.	14.4	8.6
Plr-45	72.4	43.3
Plr-63	26.8	16.0

Figure 3-5. Recombinant proteins are expressed from the vector pSW027 and retain GAPDH enzymatic activity in an *E. coli* host. Cytoplasmic lysates of *E. coli* were electrophoresed on a reducing SDS-10% polyacrylamide gel and stained with Coomassie brilliant blue to visualize proteins. Plr lane contains *E. coli* χ2602 (pSW025), vector lane contains *E. coli* χ2602 (pSW026), vector + Ω contains *E. coli* χ2602 (pSW027), Plr-53 lane contains *E. coli* χ2602 (pSW053), Plr-45 lane contains *E. coli* χ2602 (pSW045), and Plr-63 lane contains *E. coli* χ2602 (pSW063). GAPDH specific activities of each protein lysate are indicated in the adjacent table.

100.5, 72.4, 26.8 and 14.4 μM NADH min^{-1} mg $extract^{-1}$, respectively, in contrast to the background activity of 1.7 μM NADH min^{-1} mg $extract^{-1}$ for both *E. coli* χ2602 (pSW026) and *E. coli* χ2602 (pSW027). Therefore, *E. coli* expressing recombinant Plr had 60-fold higher activity over vector background as compared to 43.3-fold increase for Plr-45, 16.0-fold for Plr-63, and an 8.6-fold increase for cells expressing recombinant *B. stearothermophilus* GAPDH. These results verified that the expressed products of pSW045, pSW063, and pSW053 all possessed glycolytic activity and the genes encoding these proteins were therefore candidates for gene replacement of *plr* on the strain 64/14 chromosome.

Introduction of the gene encoding the *B. stearothermophilus gap* into strain 64/14. One approach taken to generate as isogenic mutant strain was to attempt to replace *plr* on the strain 64/14 chromosome with the *B. stearothermophilus gap* gene which encodes a non-plasmin binding GAPDH molecule. Linearized plasmid pSW053, which contains the *B. stearothermophilus gap* gene flanked by strain 64/14 sequences, was electroporated into strain 64/14. Circular pSW053 was electroporated separately as a control for integration of plasmid DNA into the chromosome. Kanamycin resistant isolates were initially screened for the presence of *B. stearothermophilus* GAPDH expression. Proteins in bacterial whole cell lysates were resolved by

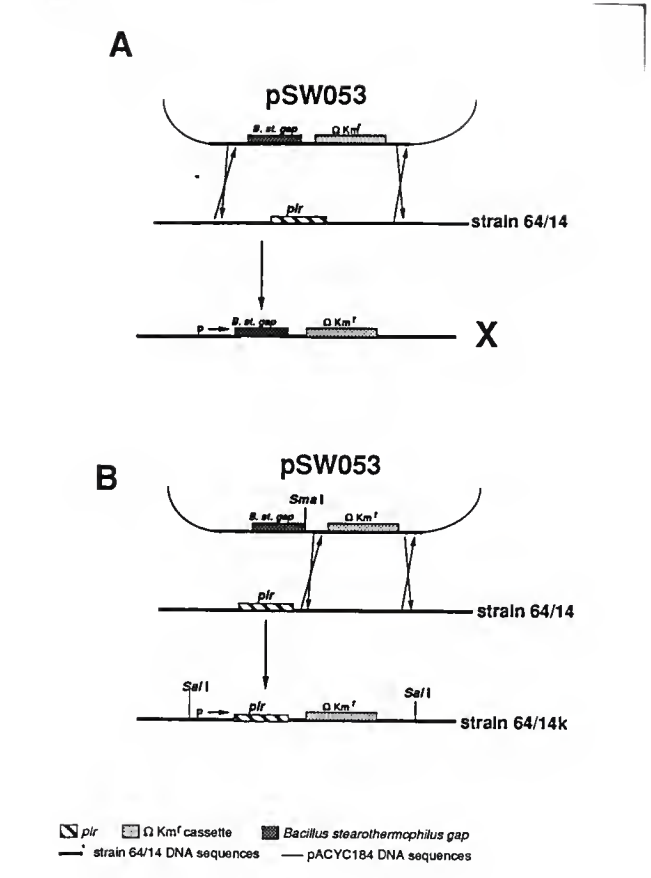


Figure 3-6. Potential recombination events resulting from electroporation of linear pSW053 into strain 64/14. A double crossover event by homologous recombination indicated by the arrows could result in replacement of the plr gene with the *B. stearothermophilus gap* and the Ω Kmr cassette (A), or integration of the Ω Kmr cassette downstream of plr, generating strain 64/14k (B).

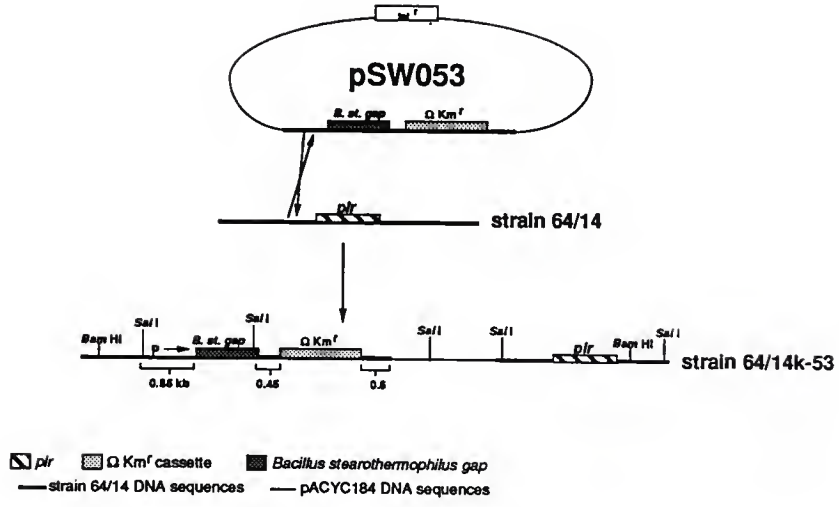


Figure 3-7. Introduction of circular pSW053 DNA into strain 64/14 yielded strain 64/14k-53. A single crossover via homologous recombination could have potentially occurred within the three regions of homology indicated by kilobase length in the figure. All transformants examined were a result of the crossover event depicted in the figure and yielded strain 64/14k-53.

electrophoresis on polyacrylamide gels, stained for protein with Coomassie brilliant blue, and inspected visually for the expression of a unique protein migrating at ~37 kDa. Approximately 40% of the isolates transformed with the circular plasmid, subsequently referred to as strain 64/14k-53, were cointegrates and had a well expressed ~37 kDa protein present in lysates (see lane 2 of figure 3-6). This protein was absent in all Km^r isolates into which linear pSW053 DNA had been introduced. These kanamycin resistant transformants were designated as strain 64/14k.

A double crossover event leading to integration of linear pSW053 DNA into strain 64/14 could have occurred 5' to the *B. stearothermophilus gap* and 3' to the Ω cassette of pSW053 with the corresponding homologous regions on the bacterial chromosome resulting in replacement of *plr* with *B. stearothermophilus gap* and the Km^r gene. Alternatively the recombination may have taken place directly 5' and 3' to the Ω cassette with the corresponding region 3' to *plr* on the chromosome resulting in isolates which were kanamycin resistant but retained *plr* instead of the *B. stearothermophilus gap* (see figure 3-7). Inspection of protein profiles of streptococcal lysates indicated that only the latter event occurred. To verify this possibility, DNA hybridization analysis was performed. Chromosomal DNA from several isolates was digested with *Sal*I and yielded a 5.5 kb band when incubated with the Ω cassette probe, which was

consistent with the fragment size predicted for integration of the Ω cassette downstream of *plr* (data not shown). Additionally, the DNA sequence extending from the 3' end of *plr* into the downstream region was determined for two of these isolates revealing an intact *plr* gene. These results verified that a double crossover event occurred in the region downstream of *plr* on the chromosome of strain 64/14k with regions of homology on plasmid pSW053 lying both 5' and 3' relative to the Ω cassette.

The integration of circular pSW053 can occur in one of three regions of homology with DNA sequences flanking *plr* in strain 64/14. To determine which of these event(s) had occurred in strain 64/14k-53, DNA hybridization analysis was performed on chromosomal DNA isolated from six 64/14k-53 isolates. DNA digested with *SalI* and transferred to nylon membranes was incubated with either a *plr* probe or a Ω cassette probe (figure 3-8). Digestion of DNA from all six isolates and wild-type strain 64/14 yielded a 3.3 kb hybridizing band with the *plr* probe, consistent with the predicted size fragment resulting from integration of the region 5' to *gap* on pSW053 into the homologous region 5' to *plr* on the chromosome. If the event occurred 5' or 3' to the Ω cassette on pSW053 with the region 3' to *plr* on the chromosome, then predicted size of the hybridizing fragments would have been 6.8 kb and 3.9 kb, respectively.

As expected, DNA from strain 64/14 did not hybridize with the Ω cassette probe whereas the DNA from the

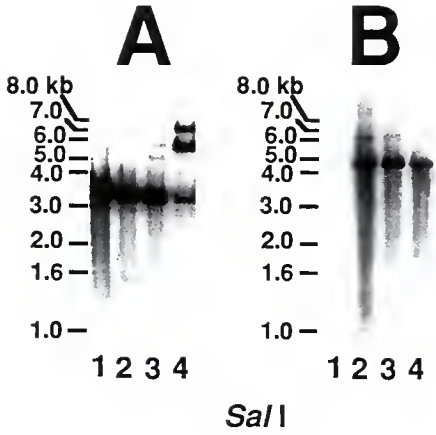


Figure 3-8. DNA hybridization analysis of chromosomal DNA from strain 64/14k-53 transformants confirmed integration of pSW053. DNA was digested with *Sal*I, electrophoresed on duplicate 0.7% agarose gels, and transferred to nylon membranes. The membranes were then reacted with a [^{32}P]dCTP labeled probe consisting of either the *plr* ORF (A), or the 2.3 kb Ω cassette (B). Membranes were hybridized and washed at room temperature, and subjected to autoradiography. Lanes 1 contain strain 64/14 chromosomal DNA, and lanes 2,3, and 4 contains chromosomal DNA from representative 64/14k-53 isolates.

transformants yielded a 4.5 kb band. Consistent with the above results, this fragment is the size predicted for the integration event occurring 5' to the *gap* gene of pSW053 and 5' to *plr* on the chromosome in contrast to fragments of 6.5 kb and 2.9 kb in length predicted for the two other possible integration events. Additionally, chromosomal DNA from 64/14 and 64/14k-53 was digested with *Nco*I and reacted with a probe consisting of the *B. stearothermophilus gap* ORF. Only the DNA from the 64/14k-53 transformants hybridized with the *B. stearothermophilus gap* probe (data not shown) indicating the presence of the gene on the bacterial chromosome as expected from the observed expression of a ~37 kDa protein in lysates of the transformants. These results indicated that a single crossover event occurred between the region 5' to *plr* on the chromosome and the region 5' to the *B. stearothermophilus gap* on pSW053 (see figure 3-7) resulting in a 5' to 3' gene order of *B. stearothermophilus gap*, Ω cassette, and *plr* on the strain 64/14k-53 chromosome.

The integration of the *B. stearothermophilus gap* at this location makes it theoretically possible for a second crossover event to take place in strain 64/14k-53 at either region of homology 5' or 3' to the Ω cassette on pSW053 with the homologous strain 64/14 endogenous sequences. The former scenario would lead to excision of *plr*, the Ω cassette, and all of the integrated plasmid sequences while the latter possibility would result in excision of only *plr* and the

plasmid sequences. Serial passage of strain 64/14k-53 in culture without kanamycin was performed in an attempt to allow a second crossover event to occur, and bacteria were then screened for excision of *plr* and the Ω cassette. After allowing bacteria to grow for a calculated 112 generations, all isolates screened remained kanamycin resistant indicating that the Ω cassette was still present. Furthermore, bacterial lysates from several of the isolates were examined by SDS-PAGE, and staining of the gels with Coomassie brilliant blue indicated that the 37-kDa protein was present. This result indicated that the second crossover had not taken place and suggested the *B. stearothermophilus gap* may not be able to replace *plr*. It was possible that either the *B. stearothermophilus* GAPDH did not posses sufficient enzymatic activity to replace Plr or that the Bacillus enzyme was not functional in the streptococcal host.

It is unlikely that the failure to replace *plr* with the *gap* of *B. stearothermophilus* can be explained by problems with the vector. The streptococcal DNA sequences 5' to the *gap* ORF of pSW053 were of sufficient size for half of a double crossover event to occur when linear pSW053 DNA was introduced into the streptococci. Furthermore, translational problems due to codon bias of the streptococcal tRNAs could be ruled out by the observed expression of the *B. stearothermophilus* GAPDH in strain 64/14k-53. The *B. stearothermophilus* GAPDH had 7-fold less activity compared to recombinant Plr when these proteins were expressed in *E.*

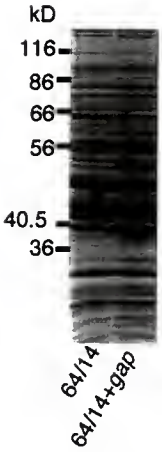
coli. Therefore it was possible that there may not have been sufficient activity of this recombinant GAPDH to replace Plr in metabolically active streptococci. Alternatively, there could have been problems with folding of the protein or with multimerization that resulted in an inactive *B. stearothermophilus* GAPDH in the heterologous host.

To address whether strain 64/14k-53 contained a functional *B. stearothermophilus* GAPDH a soluble lysate prepared from strain 64/14k-53 was assayed for GAPDH enzymatic activity and compared to a lysate of wild-type strain 64/14 (figure 3-9). The strain 64/14 lysate had 22.0 μM NADH min^{-1} mg $extract^{-1}$ whereas the 64/14k-53 lysate yielded 13.5 μM NADH min^{-1} mg $extract^{-1}$. This result suggested that although the recombinant *B. stearothermophilus* GAPDH possessed glycolytic activity in an *E. coli* host, the enzyme may not be functional in the streptococcal host.

The above results using pSW053 demonstrated that the vector pSW027 contained DNA sequences required for successful chromosomal integration.

Introduction of 3' end *plr* mutations into strain 64/14.

Using the vector pSW027, two different 3' end mutations of *plr*, *plr-45* and *plr-63*, were individually introduced via double crossover events into strain 64/14 resulting in successful mutagenesis of wild-type *plr*. Mutant strains expressed Plr-45, which contains a C-terminal leucine residue substituted for the lysine of native Plr (see Chapter 2) or



Sample	GAPDH activity (μM NADH min^{-1} mg extract^{-1})
64/14	22.0
64/14+*gap*	13.5

Figure 3-9. Comparison of GAPDH activity of cytoplasmic lysates from strain 64/14 and strain 64/14k-53. Protein lysates were assayed for GAPDH activity (see Materials and Methods of Chapter 2) as well as electrophoresed on a reducing SDS-10% polyacrylamide gel and stained with Coomassie brilliant blue to visualize the proteins. Lane 1 contains strain 64/14, and Lane 2 contains strain 64/14k-53. *B. stearothermophilus* GAPDH migrates at 37 kDa. GAPDH specific activities of each protein lysate are indicated in the adjacent table.

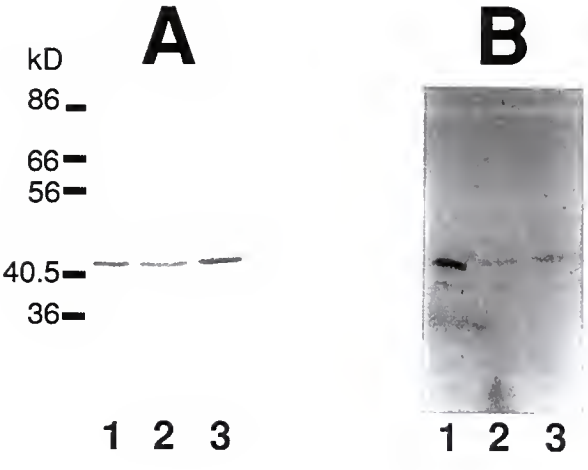


Figure 3-10. Recombinant C-terminal Plr mutations, Plr-45 and Plr-63, have reduced plasmin binding activity. Samples were electrophoresed on duplicate reducing SDS-10% polyacrylamide gels. One gel was stained with Coomassie brilliant blue to visualize proteins (A). Proteins resolved on the other gel were transferred to a nitrocellulose membrane, blocked, and reacted with [^{125}I]plasmin (B). Lanes 1 contain Plr, lanes 2 contain Plr-45 which has the C-terminal lysine residue substituted with a leucine, and lanes 3 contain Plr-63 with the C-terminus consisting of residues lysine, glycine and leucine.

Plr-63, which has a glycine and a C-terminal leucine residue fused to the C-terminal lysine residue of wild-type Plr. Plr-63 was expressed as a soluble protein, and similar to Plr-45, was purified by NAD^+ affinity chromatography. Plr-63 had the same mobility as Plr-45 when analyzed by SDS-PAGE (figure 3-10). Assessment of plasmin binding as shown in figure 3-10B revealed that Plr-45 and Plr-63 have reduced plasmin binding activity compared to Plr. Furthermore, these recombinant proteins expressed in *E. coli* demonstrated greater GAPDH activity than the *B. stearothermophilus* GAPDH expressed in *E. coli*. The linearized plasmids pSW045 and pSW063 were electroporated individually into strain 64/14, and selection of Km^r transformants was performed. The genes encoding Plr-45 and Plr-63 contain mutations at the 3' end of the *plr*, thus the 5' end crossover event can occur anywhere 5' to the mutation within the homologous sequences (figure 3-11). The presence of the desired *plr* mutations in selected isolates was proven by both DNA hybridization analyses of chromosomal DNA and DNA sequence analyses of the mutagenized regions. Integration of the homologous regions of pSW045 and pSW063 of DNA at the *plr* locus was confirmed in the two strains by analyzing *SalI* digested chromosomal DNA individually with a *plr* and a Ω cassette probe (figure 3-12). Analysis of strain 64/14 DNA yielded a 3.3 kb band when hybridized with the *plr* probe, whereas the probe hybridized with a 5.5 kb fragment in all four pSW045 isolates tested

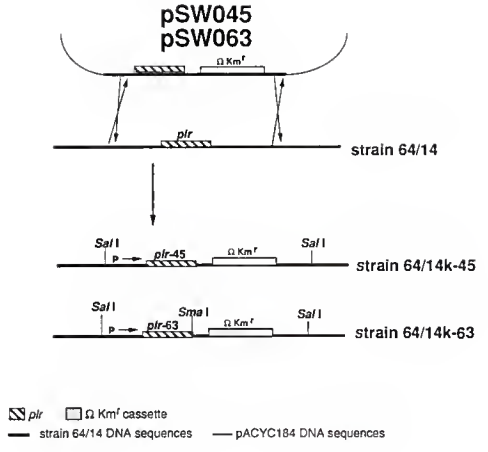


Figure 3-11. Electroporation of linear pSW045 and pSW063 DNA into strain 64/14 resulted in successful mutagenesis of *plr*. Linear plasmids were introduced individually into strain 64/14 and integrated into the streptococcal chromosome via the homologous recombination event depicted in the diagram. These events yielded the mutant strains 64/14k-45 and 64/14k-63.

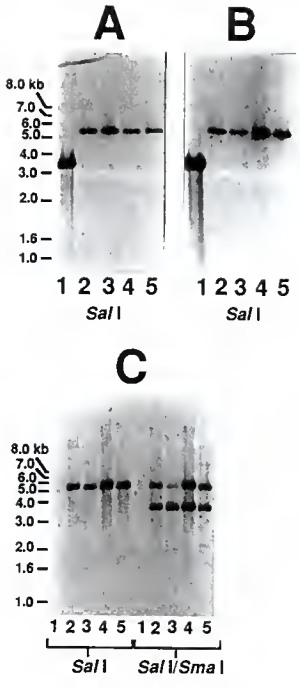


Figure 3-12. DNA hybridization analysis of chromosomal DNA from strains 64/14k-45 and 64/14k-63 transformants indicated integration of linear DNA via double crossover events. DNA was digested with either *Sal*I or a *Sal*I/*Sma*I double digest as indicated in the figure, electrophoresed on 0.7% agarose gels, and transferred to nylon membranes. The membranes were then reacted with a [^{32}P]dCTP labeled probe consisting of either the *plr* ORF (A) and (B), or the 2.3 kb Ω cassette (C). Membranes were hybridized and washed at room temperature, and subjected to autoradiography. In panel (A) lanes 1 contain strain 64/14 chromosomal DNA, and lanes 2,3, and 4 contains chromosomal DNA from representative 64/14k-45 isolates. In panels (B) and (C) lanes 1 contain strain 64/14 chromosomal DNA, and lanes 2,3, 4 and 5 contains chromosomal DNA from representative 64/14k-63 isolates.

(figure 3-12C). The 5.5 kb fragment is the predicted size of the wild-type fragment plus the 2.3 kb Ω cassette. Additionally, the presence of the Ω cassette in isolates transformed with pSW045 was verified by DNA hybridization analysis when *Sal*I digested chromosomal DNA was reacted with a Ω cassette probe and revealed the expected 5.5 kb band (data not shown).

Chromosomal DNA from strain 64/14 and isolates transformed with pSW063 were either *Sal*I or *Sal*I/*Sma*I digested and reacted with a Ω cassette probe shown in figure 3-12C. The *Sal*I digests yielded a 5.5 kb fragment in transformants (figure 3-12B) consistent with correct integration of the Ω cassette. The *Sal*I/*Sma*I double digested DNA samples were reacted with the Ω cassette probe and a 4.0 kb hybridizing fragment was identified for the transformants. The 1.5 kb difference was accounted for by a DNA fragment from the *Sal*I site upstream of *plr*-63 to the *Sma*I site at the 3' end of the *plr*-63 ORF. The 5.5 kb hybridizing fragment in figure 3-9C represents incompletely digested DNA. DNA from strain 64/14 did not react with the Ω cassette probe as expected. These data demonstrated that the Ω cassette integrated into the desired location without any extraneous vector DNA, and at least for pSW063 transformants, the crossover probably occurred 5' to the stop codon of *plr-63* suggesting these bacteria harbor the *plr*-63 mutation in place of wild-type *plr*. The predicted changes in the 3' ends of

plr in these strains was confirmed by DNA sequence analysis. Bacteria containing *plr*-45 and *plr*-63 were designated as strain 64/14k-45 and strain 64/14k-63, respectively.

Mutanolysin extracts of strains 64/14k, 64/14k-53, 64/14k-45, and 64/14k-63 were prepared as described in the Materials and Methods of Chapter 1. The 41-kDa proteins in these extracts were assessed for plasmin binding ability by the ligand blot assay to demonstrate that the *plr-45* and *plr-63* mutations introduced into the streptococci yielded expressed products that were reduced in plasmin binding, as was observed for the recombinant proteins expressed in *E. coli*. Plr and the mutant Plr proteins migrated at ~Mr 41 kDa on the Commassie brilliant blue stained polyacrylamide gel and corresponded to the immunoreactive proteins identified with anti-Plr antibody on the Western blot in figure 3-13. Both Plr-45 and Plr-63 show reduced plasmin binding ability compared to Plr in the ligand blot of figure 3-7, reflecting the results previously shown for these recombinant proteins expressed in *E. coli*.

Plasmin binding analysis of strain 64/14 mutants. The isogenic strains 64/14k-45 and 64/14k-63 were assayed for the ability to bind plasmin and compared to to strain 64/14k and wild-type strain 64/14 using several in vitro binding assays. Strain 64/14k was included to control for any effects of the Ω Km^r cassette inserted downstream of the *plr* locus and/or expression of the kanamycin resistance gene products. Strain

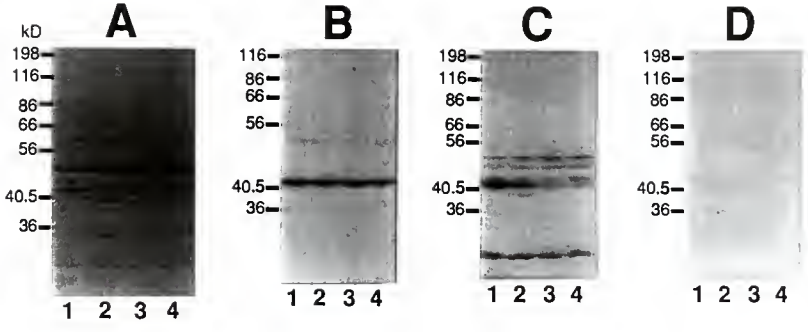


Figure 3-13. Derivatives of strain 64/14 containing *plr* mutations express plasmin binding deficient Plr molecules. Mutanolysin extracts were electrophoresed on quadruplicate reducing SDS-10% polyacrylamide gels. One gel was stained with Coomassie brilliant blue to visualize proteins (A). Proteins resolved on the other three gels were transferred to nitrocellulose membranes, blocked, and reacted with either anti-sPlr antibody (B) [^{125}I]plasmin(C) or [^{125}I]glu-plasminogen (D). Lanes 1 contain mutanolysin extract from strain 64/14k, lanes 2 contain extract from strain 64/14k-53, lanes 3 contain extract from strain 64/14k-45, lanes 4 contain extract from strain 64/14k-63.

64/14k-53, which expresses Plr and *B. stearothermophilus* GAPDH was assayed as well to examine whether an additional GAPDH, should it have surface localization, might alter plasmin binding.

Multiple sets of experiments using radiolabeled plasmin were performed and yielded consistent results. Data are presented for a representative set of assays for strain 64/14 derivatives grown overnight under antibiotic selection as well as streptococci grown in THY broth alone (Table 3-3B). The Ω cassette remained stable without selection thereby allowing any differences in plasmin binding due to effects of the antibiotic and/or expression of the Km^r gene product on growth, cell size, or surface composition to be examined. In the experiment presented in Table 3-3A strain 64/14k bound a maximum of 44.1% of the labeled-plasmin offered, and the other Km^r isogenic strains did not differ in their ability to bind plasmin. There was also no difference in labeled-plasminogen binding activity among 64/14k, which expresses wild-type Plr, and the other Km^r strains (<5% of the total plasminogen counts offered). *E. coli* χ2602 was assayed as a negative control (bound <10% of the total plasmin counts offered). The plasmin binding capacity of wild-type strain 64/14 was slightly higher than the streptococcal strains containing the Ω cassette if the Km^r bacteria were grown under selection. However, when antibiotic pressure was removed during growth these strains bound equivalent amounts

of plasmin as strain 64/14. Therefore, the differences in plasmin binding potential using bacteria grown with and without selection can be attributed either indirectly or directly to effects of kanamycin and not to the presence or position of the Ω cassette downstream of *plr*. These data revealed that mutations of the *plr* gene, which reduced the plasmin binding activity of Plr in vitro, have no effect on the ability of whole bacteria to bind pre-formed plasmin.

In addition to the above studies using radiolabeled plasmin, the streptococcal strains were tested for the ability to bind unlabeled plasmin to control for any effects that radiolabeling of the protein may have on the interaction with the bacteria. This assay requires that the cell-bound plasmin retain proteolytic activity in order to cleave the chromogenic substrate S-2251. There was greater variability in plasmin binding among isolates as assessed by measuring enzymatic activity compared to using radiolabeled plasmin. However, consistent with the binding data obtained using radiolabeled ligand, there was no significant difference in substrate hydrolysis for strain 64/14k compared to strains harboring Plr mutations and expressing the Km^r gene (see Table 3-3C and D). When Km^r derivatives were grown without antibiotic selection and compared to wild-type strain 64/14, the surface bound-plasmin activities were equivalent as observed for the assays using radiolabeled ligand. Similarly, strain 64/14k, containing wild-type *plr*, and the *plr* isogenic derivatives had reduced binding capacity when

Table 3-3. Analysis of plasmin binding for group A streptococcal strain 64/14 and strain 64/14 mutants using radiolabeled plasmin in (A) and (B), unlabeled plasmin in (C) and (D), and bacteria grown in 30% human plasma in (E).

A. Bacteria were grown without selection.

Sample	% Bound Plasmin[a]	% Bound Plasminogen[a]
64/14	33.7	3.4
64/14k	30.5	3.0
64/14k-53	27.3	3.7
64/14k-45	27.2	2.0
64/14k-63	28.9	2.0
χ2602	8.2	2.1

[a]cpm offered: ^{125}I plasmin 39460±156 (background act 2553±85)
^{125}I plasminogen 37635±1284 (background act 1993±113)
n=2, data are presented as the mean

B. The kanamycin resistant strains were grown overnight under selection.

Sample	% Bound Plasmin[a]	% Bound Plasminogen[a]
64/14	56.1	8.7
64/14k	44.1	3.4
64/14k-53	42.4	4.6
64/14k-45	42.4	2.5
64/14k-63	41.2	4.0
χ2602	6.3	1.8

[a]cpm offered: ^{125}I plasmin 47258±148 (background act 3517±66)
^{125}I plasminogen 45697±71 (background act 2572±59)
n=2, data are presented as the mean

Table 3-3 Continued.

C. Bacteria were incubated with plasminogen or pre-formed plasmin and activity assessed by hydrolysis of S-2251. Kanamycin resistant strains were grown under selection.

Sample	S-2251 hydrolysis after incubation with: Plasmin[a] (A_{405} nm)	Plasminogen[b] (A_{405} nm)
64/14	0.61±0.00	0.21±0.03
64/14k	0.30±0.05	0.08±0.03
64/14k-53	0.40±0.03	0.08±0.00
64/14k-45	0.44±0.02	0.12±0.04
64/14k-63	0.48±0.02	0.05±0.00
χ2602	0	0.02±0.01

[a]Plasmin background = 0
Spectrophotometer zeroed with 64/14 alone, no plasmin
final concentration of plasmin: 25 μg/ml plasmin (~0.6 μM)
n=3
[b]Plasminogen background= 0.029±.001
Backgrounds subtracted from data
n=3

D. Bacteria were incubated with pre-formed plasmin and activity assessed by hydrolysis of S-2251. Kanamycin resistant strains were grown under selection as indicated.

Sample	Growth conditions: THY[b] S-2251 hydrolysis (A_{405} nm[a])	THY + Kan[c] S-2251 hydrolysis (A_{405} nm[a])
64/14	0.55±0.00	-
64/14k	0.41±0.02	0.33±0.04
64/14k-53	0.85±0.03	0.37±0.02
64/14k-45	0.71±0.02	0.36±0.01
64/14k-63	0.81±0.17	0.23±0.02
χ2602	0	-

[a]Plasmin background= 0.065±0.038
Background subtracted from data
[b]THY, Todd-Hewitt yeast extract broth, n=2
[c]Bacteria grown in THY containing 350 μg/ml kanamycin, n=2

Table 3-3 Continued.

E. Bacteria were grown in 30% human plasma, washed, and incubated with S-2251 to assess surface bound plasmin activity.

Sample	S-2251 hydrolysis ($A_{405\ nm}$[a])
plasma only	0.11±0.03
64/14	0.52±0.03
64/14k	0.51±0.07
64/14k-53	0.51±0.05
64/14k-45	0.44±0.01
64/14k-63	0.60±0.13

[a]Spectrophotometer zeroed with strain 64/14 grown in THY alone.
Samples were normalized for growth by suspending bacteria to an equivalent O.D. 600nm.
n=3

grown with antibiotic pressure indicating that the differences are due to the antibiotic. Therefore, these assays revealed no differences in plasmin binding among strains 64/14k-45 and 64/14k-63 containing *plr* mutations, strain 64/14k-53 which coexpresses *B. stearothermophilus gap* and *plr*, and strain 64/14k.

Wang et al. hypothesized that in addition to binding preformed plasmin, group A streptococci can utilize an alternate mechanism to capture plasminogen and plasminogen activator activity on the bacterial surface (Wang et al., 1994). To determine if strains with mutations in *plr* differ from wild-type strain 64/14 in the ability to capture plasmin activity via this mechanism or differ in plasmin binding capacity when exposed to an environment more representative of in vivo conditions, the mutant strains were also tested using a third technique. The assay allows assessment of the plasmin activity which has been captured by the bacteria during growth in the presence of human plasma which is measured by paranitroanilide release upon hydrolysis of the substrate S-2251 (Table 3-3E). In one representative experiment, values of 0.52, 0.51, 0.51, 0.44, and 0.60 A_{405nm} were obtained for strains 64/14, 64/14k, 64/14k-53, 64/14k-45, and 64/14k-63, respectively. The results concurred with the previous assays demonstrating no significant differences amongst these streptococcal strains for surface plasmin/ plasminogen activator activity.

The results of these three techniques show that Plr mutations in the C-terminus, which show reduced plasmin binding in vitro, do not result in a reduction in the ability of whole streptococci expressing the mutated Plr molecules to bind plasmin. These findings indicate that other structures can mediate plasmin binding, and it is possible that there are multiple plasmin binding structures on the streptococcal surface. Furthermore, Plr may not be functioning as a plasmin receptor in wild-type streptococci.

The effects of protease treatment of the plasmin binding activity of strain 64/14. The role of surface proteins and the contribution of C-terminal lysine residues of such proteins to the plasmin binding phenotype of strain 64/14 was examined. Bacteria were treated with either trypsin or carboxypeptidase B, an enzyme that hydrolyzes C-terminal basic residues, and then assayed for plasmin binding activity using radiolabeled ligand (see figure 3-14). Control samples were either incubated in buffer alone, protease inactivated with inhibitor, or protease inhibitors alone. Strain 64/14 treated with trypsin for 1 hr lost up to 60% of its plasmin binding ability indicating that trypsin-sensitive surface proteins component(s) contribute significantly to the interaction. Carboxypeptidase B treatment of cells resulted in a loss of 44% plasmin binding ability after 4 hr of exposure to the protease. This result would suggest that C-terminal lysine or arginine residues are at least partially

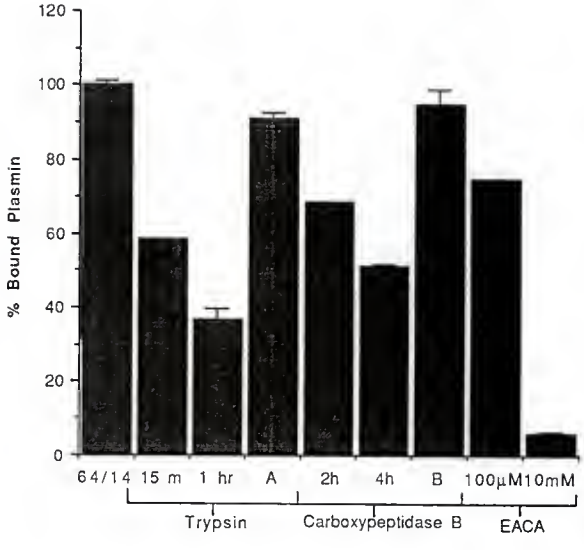


Figure 3-14. Effects of protease treatment and EACA on the ability of strain 64/14 to bind plasmin. Bacteria were incubated in the presence of protease or inhibitor-inactivated protease for the times indicated. Bacteria were then washed and incubated with [^{125}I]plasmin. Quantification of bound plasmin was calculated as follows; cpm bound by the bacteria-background cpm of ligand alone/ total cpm of ligand offered X 100, and are presented as the percentage of binding activity relative to untreated strain 64/14. Strain 64/14 bound 47.3% of the total ligand offered and is set at 100% binding activity. A=aprotinin, B=benzylsuccinic acid. n=3.

responsible for the plasmin binding phenotype of strain 64/14. Lysine interactions have previously been hypothesized to be involved in the binding of plasmin to the bacteria because plasmin could be inhibited from binding by the presence of free lysine or epsilon aminocaproic acid (EACA) in a concentration dependent manner (Broeseker et al., 1988). In these assays, EACA concentrations of 100 μM and 10 mM inhibited binding by up to 30% and 98%, respectively. Cells first treated with either trypsin or carboxypeptidase B and then incubated with plasmin in the presence of 10 mM EACA yielded equivalent results as untreated cells incubated with the EACA (data not shown), indicating that although the proteases used in these experiments cannot eliminate all plasmin binding, essentially all of the plasmin binding ability may be lysine dependent. Trypsin-treated cells were subsequently incubated with carboxypeptidase B, but plasmin binding was not further reduced by the carboxypeptidase B treatment (data not shown).

Examination of Plr from trypsin treated strain 64/14. Trypsin treatment of strain 64/14 resulted in a substantial decrease in plasmin binding. To examine whether Plr was affected by exposure of the cell surface to this protease, proteins in mutanolysin extracts from trypsin treated cells and an untreated control were analyzed for Plr degradation products using anti-Plr polyclonal antibody by Western

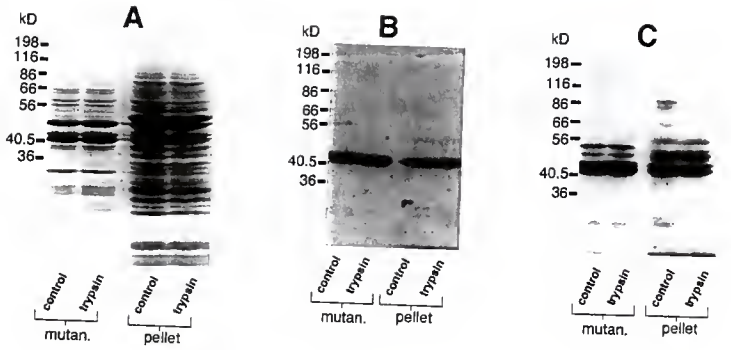


Figure 3-15. Trypsin treatment of streptococcal strain 64/14. Trypsin treatment of strain 64/14 was performed as described in the Materials and Methods. Mutanolysin extracts (mutan.) were prepared from each sample. Protein extracts and protoplast pellet fractions were electrophoresed on triplicate reducing SDS-10% polyacrylamide gels. One gel was stained with Coomassie brilliant blue to visualize proteins (A). The other two gels were transferred to nitrocellulose membranes, which were blocked and reacted with either anti-sPlr antibody (B), or probed with [^{125}I]plasmin (C). Lanes are as indicated in the figure.

immunoblot analysis, and plasmin binding ability by the ligand blot assay. The mutanolysin extracts and the remaining protoplasts were subjected to SDS-PAGE on triplicate gels (see figure 3-15). The gel stained with Coomassie brilliant blue in panel A shows no differences in relative quantities of Plr in either the mutanolysin extracts or the pellet fractions. When immobilized proteins were probed with antibody, only a single band at ~41 kDa was detected in both the control and protease treated samples, suggesting that significant degradation of Plr had not occurred. Furthermore, Plr retained wild-type levels of plasmin binding in protease treated bacteria as shown in the ligand blot of panel C. These results demonstrated that although trypsin causes a reduction in plasmin binding of strain 64/14, the molecular weight of Plr as well as its ability to bind plasmin in vitro was unaffected by trypsin treatment of the bacterial surface.

A C-terminal lysine residue of Plr was shown to be necessary for wild-type levels of plasmin binding in vitro in Chapter Two. These mutations, when introduced at the *plr* locus in strain 64/14 resulted in no change in plasmin binding of the whole bacteria. Therefore, if Plr is binding plasmin at the cell surface, it would be through a mechanism other than a C-terminal lysine residue and a domain which is not accessible to trypsin degradation.

Surface iodination of strain 64/14. To examine the surface localization of Plr, strain 64/14 was incubated in the presence of [^{125}I]Na and lactoperoxidase to iodinate exposed components. The mutanolysin extract and the corresponding cell pellet prepared from the labeled cells was examined by SDS-PAGE. Commassie brilliant blue staining revealed a typical mutanolysin extract profile of proteins, containing a substantial quantity of Plr migrating at ~Mr 41 kDa (figure 3-16). Multiple reactive bands were identified, however substantial labeling of a 41-kDa protein was not identified. It is evident that a significant quantity of Plr, a major determinant component of the mutanolysin extract, was not radiolabeled.

Phosphoglycerate kinase (PGK) assays. In order to examine whether mutanolysin extracts of group A streptococci contain cytoplasmic proteins, several different extracts of strain 64/14 were examined for another glycolytic enzyme, PGK, using a spectrophotometric assay. The data show a range of a 1.2 to 6-fold increase in PGK activity of cytoplasmic lysates relative to the activity of the corresponding mutanolysin extract preparations (see Table 3-4). PGK specific activities in mutanolysin extracts prepared in PBS increased from 0.3 to 0.4 μM NAD^+ min^{-1} mg protein $extract^{-1}$ at 1.5 and 3.5 hr time points, respectively. Similarly, when

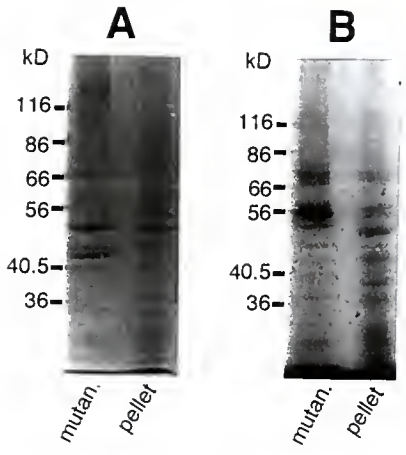


Figure 3-16. Iodination of the strain 64/14 cell surface. Strain 64/14 was labeled with [^{125}I]Na using lactoperoxidase. A mutanolysin extract (mutan.) was then prepared from the bacteria. Protein extract and the protoplast pellet fraction were electrophoresed on duplicate reducing SDS-10% polyacrylamide gels. One gel was stained with Coomassie brilliant blue to visualize proteins (A). The other gel was dried on Whatman filter paper and exposed to photographic film for 5 days. Lanes are as indicated in the figure.

Table 3-4. A comparison of phosphoglycerate kinase activity of group A streptococcal strain 64/14 mutanolysin extracts and the corresponding cytoplasmic extracts.

Sample	PGK sp act[a] mutan extract	PGK sp act[a] cytoplasm	Buffer, Time[b]	Ratio of cyto/ extract act[c]
1	0.3	1.4	PBS, 1.5 h	5.9
2	0.2	0.2	PBS, 1.5 h	1.2
3	0.1	0.3	30% raf., 1.5 h	1.9
4	0.3	1.7	PBS, 1.5 h	5.9
4	0.4	1.7	PBS, 3.5 h	4.0
5	0.4	1.3	30% raf., 1.5 h	3.7
5	0.8	1.3	30% raf., 3.5 h	1.7

[a] μM NAD^+ min^{-1} mg protein $extract^{-1}$

[b] PBS or PBS containing 30% raffinose (raf.), hours cells incubated with mutanolysin

[c] cytoplasmic sp act / mutanolysin extract sp act

strain 64/14 mutanolysin extracts were prepared in PBS containing 30% raffinose the activity increased with time from 0.4 to 0.8 μM NAD^+ min^{-1} mg protein $extract^{-1}$ at 1 hr and 3.5 hr, respectively. The addition of 30% raffinose during the mutanolysin extraction did not appear to have an effect on the amount of PGK activity found in the mutanolysin extracts or the increase of the PGK activity over time. The presence of a second glycolytic enzyme activity, PGK, identified in mutanolysin extracts of group A streptococci suggests that cytoplasmic enzymes may contaminate these preparations either by cell leakage or cell lysis during degradation of the peptidoglycan cell wall. Alternatively, it is possible that there are multiple glycolytic enzymes associated with the cell wall. However, the results indicate that the presence of a protein in a mutanolysin extract should not be the sole criterium to qualify it as a cell wall-associated protein.

Discussion

Group A streptococci bind plasmin with a high affinity (K_d 10^{-10} M) (Broeseker et al., 1988). This binding interaction is specific for molecules of plasmin and lys-plasminogen (Broder et al., 1989). The ability of free lysine or lysine analogs to inhibit or compete plasmin off the bacterial surface suggests that the lysine binding sites

(LBS's) of plasmin(ogen) may be involved in the interaction, possibly with C-terminal lysine residues of surface proteins mediating the binding. The concentration of EACA, a lysine analog, which inhibits binding was consistent with the high affinity LBS of kringle 1 interacting with surface structures, although it is possible that interactions take place via other domains of plasminogen which are conformationally altered by lysine. Plasmin may bind to both surface proteins and non-proteinaceous components such as lipoteichoic acid or peptidoglycan; however, the substantial reduction in binding from protease treatment of strain 64/14 suggests a predominant role for protein. Therefore to define the role of the plasmin binding protein Plr as a plasmin receptor the mutagenesis studies in this chapter were performed.

The *plr* gene could not be inactivated, and so to circumvent this problem several alternative strategies were applied to mutagenize *plr* in strain 64/14. Mutational analysis presented in Chapter 2 yielded plasmid pRL037 containing a 3' mutation of *plr* generated by unidirectional deletion. The product, Plr-37, had reduced plasmin binding ability compared to Plr and retained GAPDH enzymatic activity in an *E. coli* host. The plasmid pSW037 contains the 3' end two-thirds of *plr-37* while pSW024 harbors the equivalent region of wild-type *plr*. These plasmids were designed as cointegrative plasmids that could potentially recombine into the streptococcal chromosome via a single homologous

recombination event. In contrast to the integration of pSW024 into strain 64/14, pSW037 did not yield viable transformants. This result may be explained by several possibilities. Plr-37 may not possess enough specific GAPDH activity to sustain growth of the organism. It is also possible that Plr-37, although soluble and glycolytically active in *E. coli*, did not fold correctly in streptococci and its enzymatic activity was thereby disrupted. Alternatively, unlike pSW024, the plasmid pSW037 does not contain any streptococcal DNA sequences extending from the 3' end of *plr*-37 up to the 5' end of the Ω cassette in pSW037 because these sequences were removed during the exonuclease III deletion procedure. Sequences within this region could potentially be necessary for efficient mRNA transcription or stability of the transcript in the streptococcal background.

The successful integration of pSW024 into the streptococcal chromosome indicated that the insertion of the Ω cassette downstream of *plr* was not a deleterious event. Therefore if the Ω cassette of strain 64/14-24 was preventing any potential downstream transcription, it must be of a non-essential gene(s). Multiple genes encoding glycolytic enzymes are often clustered together and in several species, such as *Corynebacterium glutamicum* and *Zymomonas mobilus*; transcriptional analysis has shown that these genes can be transcribed as polycistronic messages (Schwinde et al., 1993; Conway and Ingram, 1988). In several Gram-positive species, such as *Bacillus megaterium* and *Corynebacterium glutamicum*,

the *gap* gene is followed by the gene encoding phosphoglycerate kinase gene (*pgk*) and the triose phosphate isomerase gene (Schlapfer and Zuber 1992, Eikmanns, 1992). These genes lie within several hundred base pairs of each other. There are variations of this organization, however, such as in *E. coli* where *gap* B and *pgk* are clustered together, but *gap* A, another gene encoding GAPDH, is located elsewhere on the chromosome. Only a product of *gap* A has been identified as a functional GAPDH in *E. coli*. However, the glycolytic enzymes of group A streptococci have not been previously characterized at the gene or protein level. Analysis of DNA sequence data from our laboratory of approximately 1 kb of upstream and downstream DNA flanking *plr* did not identify any ORF of other glycolytic enzymes, suggesting that *plr* may not reside in the more typical prokaryotic gene arrangement.

The information gained from introducing the cointegrative plasmid pSW024 successfully into strain 64/14 was applied to the construction of pSW027 for use in the introduction of an alternative *gap* gene or *plr* mutations into strain 64/14.

Several characteristics of the *B. stearothermophilus gap* seemed to make it an ideal candidate for gene replacement. The *B. stearothermophilus* GAPDH has been cloned and sequenced (Branlant et al., 1983), and has 71% similarity and 53% identity to Plr at the predicted amino acid sequence level and 54% identity at the DNA sequence. Also important for

these studies, the protein does not bind plasmin on ligand blots as shown in figure 2-1 of Chapter 2. Therefore the *B. stearothermophilus gap* ORF was subcloned into pSW027 to yield pSW053 and transformed into *E. coli*. The 37-kDa gene product was soluble and expressed. Importantly, the protein was enzymatically active in an *E. coli* host background, although the specific activity was several-fold lower than the activity of Plr expressed in the same host strain.

Repeated introduction of linearized pSW053 DNA into strain 64/14 yielded a total of 40 Km^r colonies which expressed Plr but lacked expression of a 37-kDa protein. In contrast, screening of the Km^r isolates resulting from electroporation of circular pSW053 DNA revealed that 16 out of 41 streptococcal isolates expressed both Plr and a novel 37-kDa protein. The results of DNA hybridization analysis from six of these 16 co-expressing transformants were consistent with homologous recombination occurring within the region lying 5' to the *B. stearothermophilus gap* on pSW053 with the region 5' to *plr* on the chromosome. The crossover area of pSW053 contains approximately 800 bp of homology with strain 64/14 versus 400 bp and 600 bp for the regions 5' and 3', respectively, to the Ω cassette. The gene arrangement on the chromosome of strain 64/14k-53 is *B. stearothermophilus gap* followed by the Ω cassette and followed by *plr*. Based on extent of homology alone, the 39% frequency of this event occurring (as assessed by the DNA hybridization analysis of randomly chosen isolates which expressed the 37-kDa protein)

is close to the predicted 45% calculated frequency of occurrence, although not too far from the predicted 24% and 31% frequency for crossovers occurring within the 450 bp and 600 bp homologous regions, respectively. It is possible that the remaining 61% of isolates not expressing *B. stearothermophilus gap* represent crossover events of these other regions. The resulting 5' to 3' gene orders in such isolates are *plr*, Ω cassette, *gap;* or *plr*, *gap*, and Ω cassette for crossovers within the 450 bp and 600 bp region, respectively. These events would allow for a second crossover event to excise the *gap* gene but leave *plr* and Ω cassette remaining on the chromosome, allowing for expression of Plr and resistance to kanamycin. Although transformants lacking expression of *gap* have not been examined genetically, this hypothesis is consistent with the biochemical data suggesting that the recombinant *B. stearothermophilus* GAPDH may not have functional enzymatic activity in the streptococcal host. In contrast, the second crossover that would potentially occur in strain 64/14k-53, where *gap* precedes both the Ω cassette and *plr*, would retain antibiotic resistance but result in excision of *plr*. Passage of strain 64/14k-53 in successive cultures without antibiotic selection for 112 generations did not result in a second crossover at the 450 bp region that would have yielded a *plr* minus and kanamycin sensitive strain in the several hundred colonies screened. Furthermore, a cytoplasmic lysate of strain 64/14k-53 yielded no increase in GAPDH enzymatic activity compared to strain 64/14. This

result also indicates *B. stearothermophilus* GAPDH may not have enzymatic activity in this host and supports the hypothesis that *plr* is an essential gene.

The gene replacement strategy utilized for the introduction of *B. stearothermophilus gap* into streptococci suggested that open reading frames cloned into the *Nco* I and *Sma* I sites of pSW027 could potentially be integrated and expressed at the *plr* locus in strain 64/14. Two individual *plr* mutations, *plr-45* and *plr-63*, yielding products with reduced plasmin binding and partial GAPDH activity relative to Plr were subcloned into pSW027 to generate pSW045 and pSW063, respectively. As characterized in Chapter 2, Plr-45 contains a leucine substituted for the C-terminal lysine of Plr. Glycine and leucine are the two predicted C-terminal residues of the *B. stearothermophilus* GAPDH and are preceded by a lysine residue. It was demonstrated by analyzing Plr-63 that placement of the C-terminal lysine of Plr in an internal position, identical to that found in the C-terminus of *B. stearothermophilus* GAPDH, results in reduced plasmin binding activity of the protein. Both Plr-45 and Plr-63, expressed in *E. coli* χ2602 (pRL045) and *E. coli* χ2602 (pRL063) respectively, had enzymatic activity greater than the *B. stearothermophilus* GAPDH expressed in *E. coli* χ2602 (pRL053) although slightly less activity than wild-type Plr from *E. coli* χ2602 (pRL025). Both mutations were subsequently integrated successfully by double crossover events of linearized plasmid DNA, resulting in loss of wild type *plr*

due to replacement with *plr*-63 in strain 64/14k-63 and *plr*-45 in streptococcal strain 64/14k-45. Insertion of the selectable marker downstream of *plr* derivatives was confirmed by DNA hybridization analyses and DNA sequencing of the mutagenized regions confirmed the presence of the desired *plr* mutations. Furthermore, mutanolysin extracts prepared from mutant strains 64/14k, 64/14k-45, and 64/14k-63 were assayed for plasmin binding and consistent with the recombinant proteins expressed in an *E. coli* host, mutant Plr from either strain 64/14k-45 or strain 64/14k-63 had reduced plasmin binding compared to Plr from strain 64/14k.

Isogenic strains were compared with wild-type strain 64/14 for differences in the ability to bind plasmin by several different techniques. Two of the assays measure the binding of pre-formed plasmin. One assay uses radiolabeled plasmin as ligand and the other uses unlabeled plasmin with subsequent analysis of cell-associated proteolytic activity measured using a plasmin-specific chromogenic substrate. Strain 64/14k-45 and strain 64/14k-63 which contain C-terminal mutations of Plr consistently showed equivalent amounts of plasmin bound as strains 64/14k and strain 64/14k-53 which express wild-type Plr.

In the third assay, the capture of plasmin-like activity by bacteria grown in the presence of plasma was measured for wild-type and mutant derivatives of strains 64/14. This assay requires that the bacteria generate plasminogen activator activity and subsequently capture plasmin activity

on the surface. This plasmin activity may be captured by a mechanism independent of direct binding of preformed plasmin. Wang et al. have postulated that plasminogen activator activity can be localized to the bacterial surface by a streptokinase:plasmin(ogen) complex which may be bound via fibrinogen to M-like proteins (Wang et al., 1994). This system is somewhat analogous to plasminogen binding to platelets either directly to surface glycoprotein IIb/IIIa or via fibrin which is bound to the same molecule (Miles et al., 1986), and is also analogous to surface localization of plasminogen activator activity on eukaryotic cells by the urokinase and tPA receptors. However, consistent with the results of the other assays, there were no significant differences in surface plasmin activity among strain 64/14k expressing Plr, strain 64/14k-53 expressing both Plr and *B. stearothermophilus* GAPDH, strain 64/14k-45 and strain 64/14k-63 both of which express mutant Plr containing C-terminal alterations. Therefore C-terminal lysine residues of Plr have no apparent role in the capture of plasmin by bacteria via this additional pathway. All three techniques of assessing plasmin binding activity of streptococci performed in these studies consistently showed that expression of mutant Plr molecules with alterations of the C-terminal lysine do not affect the ability of the intact streptococci to bind wild-type levels of plasmin on the bacterial surface, even though these mutated proteins demonstrated reduced plasmin binding in vitro relative to wild-type Plr.

Therefore, Plr may not be a plasmin receptor as originally hypothesized.

It is possible that plasmin binding of surface localized Plr may be reduced in mutant strains but other plasmin binding structures may compensate for this loss of binding. Trypsin treatment of strain 64/14 resulted in a 60% decrease in plasmin binding compared to untreated bacteria or bacteria incubated with aprotinin inactivated trypsin. These results suggested that proteins play a significant role in plasmin binding although non-proteinaceous structures such as peptidoglycan or lipoteichoic acid could potentially contribute to binding. Therefore, the possibility of multiple plasmin binding structures on the surface of group A streptococci remains.

Trypsin and plasmin cleave on the carboxyl side of lysine and arginine residues in a variety of proteins. In contrast to this study, Camacho et al. found a 2- to 5-fold increase in the binding of inactivated plasmin to colonic carcinoma SW1116 cells when these cells were pretreated with either plasmin or trypsin (Camacho et al., 1989). The affinity of the novel binding sites for plasmin was equivalent to that of untreated cells (K_d 10^{-7} M) and suggested that the exposed residues may be in a similar conformation as those of untreated cells. Analogous to strain 64/14, SW1116 cells have a higher affinity for plasmin relative to glu-plasminogen. However, C-terminal lysines exposed by trypsin treatment of strain 64/14 may not be in a

conformation conducive for high-affinity interaction with plasmin or similarly, substantial cleavage of plasmin binding surface structures of strain 64/14 could potentially render newly exposed binding sites inaccessible to plasmin. This hypothesis is supported by demonstrating that trypsin-treated streptococci that were subsequently incubated with carboxypeptidase B (CPB), which specifically cleaves C-terminal lysine and arginine residues, did not display any further reduction in plasmin binding (data not shown). All remaining plasmin binding activity of the trypsin-treated bacteria is essentially abolished in the presence of 10 mM EACA which may indicate that there is still either direct or indirect involvement of the kringle regions of plasmin with the remaining streptococcal binding sites.

While proteins may mediate much of the plasmin binding activity of group A streptococci, it was possible that C-terminal lysine residues of these surface proteins are not involved in the binding interaction. Streptococci were treated with CPB to assess the importance of C-terminal lysine residues potentially present on these structures. CPB treatment reduced the ability of strain 64/14 to bind plasmin in a time dependent manner by up to 44%. This is consistent with the ability of lysine and EACA to abolish plasmin binding to the bacterial surface and further substantiates the importance of C-terminal lysines in plasmin binding protein structures.

Comparable to the results presented here, Miles et al. found that treatment of U937 cells with CPB led to a decrease in plasminogen binding of up to 67% compared to untreated cells (Miles et al., 1991). Similarly, treatment of SW1116 cells with CPB reduced plasmin binding by 50% (Camacho et al., 1989).

The inability of CPB to completely eliminate binding of strain 64/14 may indicate an alternative binding mechanism(s) or inaccessibility of C-terminal lysine residues to this enzyme. Consistent with this hypothesis, plasmin binding was eliminated when the CPB treated bacteria were incubated with plasmin and 10 mM EACA. Furthermore, plasmin binding was not further reduced when cells were treated with CPB subsequent to trypsin treatment which suggested that CPB did not cleave additional lysine residues that may have been exposed by trypsin. Together, these results demonstrated that surface proteins play a role in the ability of strain 64/14 to bind plasmin and part of the activity may be dependent upon C-terminal lysine residues.

The C-terminal lysine of Plr was shown to be required for wild-type binding activity in vitro, but it is possible that this putative plasmin receptor binds via a different mechanism when potentially surface bound.

One explanation for the mutant derivatives of strain 64/14 having equivalent plasmin binding ability to wild-type streptococci could be that the ligand binds via internal lysine residues of receptor proteins. The participation of

internal lysine residues of fibrin in directly mediating the binding of glu-plasminogen via the low affinity LBS of kringles 2 through 4 has been reported (Lucas et al., 1983). These data were consistent with the studies of Pannell et al. indicating that glu-plasminogen could bind to CPB treated fibrin and undergo activation by tissue plasminogen activator (tPA), which binds fibrin at a different site (Pannell et al., 1988). However many group A streptococci specifically bind plasmin or lys-plasminogen including strain 64/14, having little affinity for glu-plasminogen, although there are other strains which bind both plasmin and glu-plasminogen albeit with different affinities (Wang et al., 1993; Ullberg et al., 1989). Additionally, only lys-plasmin(ogen) and to a lesser extent isolated heavy chain of lys-plasmin, containing the kringle regions, could efficiently compete bound plasmin from the cell surface of strain 64/14 (Broder et al., 1989). Specificity of ligand is further demonstrated by the lack of binding by proteins that contain kringle regions homologous to those of plasmin(ogen) such as urokinase and tPA (DesJardin et al., 1989). The lack of binding of glu-plasminogen to the bacteria argues against significant contributions by internal lysine residues as does the I_{50} concentration of lysine, which is consistent with interaction of the high affinity LBS of plasmin with plasmin binding surface structures of streptococci. Furthermore, genetic mutational analysis of *plr* implicates the C-terminal lysine residue but not the penultimate lysine. A substitution of

the C-terminal lysine in Plr-45 resulted in a similar decrease in plasmin binding as a Plr mutant protein with deletion of both the C-terminal and penultimate lysines, Plr-28. Substitution of the penultimate lysine residue in Plr-46 did not alter plasmin binding activity. Additionally, wild-type and mutant Plr from mutanolysin extracts of strains 64/14k, 64/14k-53, 64/14k-45, and 64/14k-63 show no reactivity on ligand blot assay with glu-plasminogen. Because mutations of Plr which affect the C-terminal lysine still bind some plasmin on ligand blot assay, albeit greatly reduced, it is possible that there is some interaction of plasmin with other residues of Plr. However the C-terminal lysines necessary for high affinity binding are absent in the mutant strains and the wild-type levels of surface binding cannot be accounted for by this possibility alone.

Proteolytic cleavage of Plr molecules which could expose novel C-terminal lysine residues could be an alternative explanation for the plasmin binding levels seen for the mutant strains. However, streptococci analyzed in plasmin binding assays using either [^{125}I]plasmin or unlabeled plasmin and S-2251 to assess binding were grown and prepared in vitro thereby ruling out the introduction of any exogenous proteases which could potentially expose novel C-terminal lysine residues on Plr. Additionally, Plr-45 and Plr-63 from the mutanolysin extracts of mutant strains 64/14k-45 and 64/14k-63, respectively, still demonstrated reduced plasmin binding relative to Plr from strain 64/14k by ligand blot

assay. Therefore, the exposure of novel C-terminal lysine residues as the result of cleavage by any endogenous proteases can be ruled out as well.

It is possible that Plr may not be a plasmin receptor of strain 64/14 but may be localized to the bacterial surface to potentially serve alternative function(s). Thus, several experiments were performed to address the exposure of Plr on the surface. Mutanolysin extracts prepared from trypsin treated and untreated strain 64/14 showed no apparent reduction in quantity of Plr in either the mutanolysin extracts or the remaining pellet fractions of treated cells compared to the untreated control. There were also no Plr degradation fragments detectable with anti-Plr polyclonal antibody. Although the antibody might not be able to recognize all potential Plr fragments, this antibody is able to recognize multiple epitopes of Plr as determined the by Western blot analyses of the mutagenesis studies of Chapter 2. Plasmin binding also remained equivalent in treated and untreated cells indicating that regions of Plr had not been proteolytically cleaved.

Plasmin binding activity of Plr from protease treated cells could potentially be accounted for by contamination of cytoplasmic Plr into the mutanolysin extracts. The cytoplasmic Plr not accessible to trypsin may be masking partially degraded Plr in the ligand blot which could have lost plasmin binding activity. To address this, the plasmin bound to each of the respective 41-kDa band in the ligand

blot was determined by excising the bands of interest from the nitrocellulose membrane and measuring the counts per minute (cpm) using a gamma counter. When cpm were divided by the respective protein concentration of the extracts there was a 10% increase in binding activity in the trypsin treated sample. However, this increase was also present for an unrelated 45-kDa plasmin binding band present in both mutanolysin extracts on the same blot. Therefore, any potential cell leakage caused by mutanolysin treatment does not account for the equivalent levels of plasmin binding by Plr from trypsin treated and untreated bacteria.

To complement the studies of trypsin treated strain 64/14, supernatants from trypsin or V-8 protease treated bacteria were examined by Western blot using the anti-Plr antibody. There was no detectable intact or degradation products of Plr in any of the supernatants (data not shown).

Trypsin treatment of bacteria result in a 60% loss of plasmin binding, yet Plr appears unaffected either in its -Mr or its plasmin binding activity. However, resistance to trypsin does not necessarily negate its putative surface localization. The lysine and arginine substrate amino acids must be accessible for cleavage by the protease, as do other residues required for efficient substrate binding. There are examples of both trypsin resistant and trypsin sensitive surface proteins of group A streptococci. The R-antigen surface protein of group A streptococci is trypsin resistant but pepsin sensitive (Johnson, 1975). Trypsin has been used

successfully to digest surface exposed portions of M-protein to aid in the identification of the cell-wall associated regions (Pancholi and Fischetti, 1988). Recently, a putative collagen receptor was isolated from strain 64/14 by collagen affinity chromatography. Trypsin treatment of whole bacteria was able to solublize a peptide that retained collagen binding activity and which was immunoreactive with antibody raised against the purified 57-kDa protein (Visai et al., 1995). It is possible that potential surface localized Plr would not be assessable to trypsin and that the substantial reduction of plasmin binding observed after trypsin treatment is the result of degradation of other functional receptors. Alternatively, it is also possible that Plr may be localized in the cell wall and not exposed on the surface, or that Plr may not be associated with the cell wall. Definitive conclusions concerning cellular localization of Plr or its role as a plasmin receptor cannot be made based on this experiment. Interestingly, there are several high and low Mr plasmin binding proteins visible in the untreated cell pellet fraction which are absent after trypsin digestion. These may be potential plasmin receptors of strain 64/14 and is consistent with the hypothesis that multiple plasmin receptors are present on the cell surface.

Iodination of surface proteins has been used successfully to identify a putative plasminogen receptor from eukaryotic cells (Miles et al., 1991). Soluble protein extracts were prepared from radiolabeled U937 cells.

Plasminogen binding proteins were isolated from these extracts by plasminogen affinity chromatography. When strain 64/14 putative surface structures were examined by iodination of whole cells using lactoperoxidase and Na ^{125}I, labeled proteins were present in both mutanolysin extract and protoplast pellets prepared after treatment. However, there was no 41-kDa protein visible by autoradiography, although a significant quantity of the protein was visible in the mutanolysin extract as revealed by Coomassie brilliant blue staining of the gel. There are only two tryptophan residues in Plr, one towards the NH_2-terminus of the protein and the other in closer proximity to the C-terminus, yet a purified preparation of Plr labels to a high specific activity (data not shown). However, if Plr does have surface localization, either residue may not be assessable to the lactoperoxidase due to obstruction by its involvement in anchoring the protein to the surface or indirectly because of altered protein folding due to its association with the peptidoglycan. Lack of cell surface exposure of Plr could also account for the apparent absence of iodination of this protein.

In performing the above experiments, cells were usually treated with hyaluronidase to potentially maximize exposure of cell surface components to the proteases or lactoperoxidase. Western blot analysis using anti-Plr antibody of supernatant after hyaluronidase treatment of cells indicated that no Plr protein was associated with the

bacterial capsule (data not shown). This is in agreement with previous results which demonstrated that no Plr was found in supernatants after heat treatment at 80° C for 10 min, treatment with 2% SDS, hot acid or alkali treatment, nor could Plr be identified in concentrated culture media in which strain 64/14 had been grown. These results indicate that Plr is not loosely associated with the cell surface. Previous plasmin binding assays performed in our laboratory have also demonstrated no alteration in plasmin binding ability of group A streptococci with hyaluronidase treatment.

Together these experiments strongly suggest that Plr is not bound to the bacterial surface in a readily accessible state nor is it associated with the bacterial capsule.

Plr has been identified as a candidate surface receptor due to its plasmin binding activity and because the protein is present in mutanolysin extracts of all group A streptococci examined in our laboratory. Isogenic mutations of *plr* in strain 64/14 show no differences in the ability of the bacteria to capture plasmin suggesting that Plr may not be a plasmin receptor. Furthermore the additional, albeit limited, experiments performed in these studies do not provide evidence for surface localization of Plr. It is possible that cell leakage or cell lysis during mutanolysin treatment of the bacteria allows intracellular Plr to contaminate these extracts.

Pancholi and Fishetti reported a surface localized GAPDH (SDH) of group A streptococci. The isolated protein

bound fibronectin, myosin heavy chain, lysozyme, and actin by ligand blot assay and the molecule also possessed ADP-ribosylation activity in vitro (Pancholi and Fischetti, 1992 and 1993). However, similar to isolation of Plr (Broder et al., 1991), surface localization of the multi-functional GAPDH was primarily based upon the presence of the protein in extracts prepared after peptidoglycan degradation. Additionally, the authors treated whole streptococci with trypsin and reported a subsequents loss of anti-SDH antibody reactivity to the bacteria surface using a dot blot assay. However, group A streptococci often express IgG receptors on the surface which can result in non-specific antibody binding and furthermore no controls were performed for comparison in these experiments.

The muramidases lysin and mutanolysin, which degrade peptidoglycan have been used previously for solublization of well characterized surface components of group A streptococci such as M protein and IgG binding proteins (Fishetti et al., 1976; Yarnall and Boyle, 1986). However, examination of these preparations for contamination by cytoplasmic proteins has not previously been reported for group A streptococci.

Mutanolysin extracts and the corresponding cytoplasmic proteins of strain 64/14 were assayed for phosphoglycerate kinase (PGK) activity and revealed the presence of substantial enzymatic activity found in the mutanolysin extracts relative to the cytoplasmic lysate. The PGK activity in mutanolysin extracts increased with time and the

presence of 30% raffinose, which is added to potentially prevent cell lysis, had no effect on this activity. This leaves open the possibility that Plr may not be a plasmin receptor of strain 64/14 because it is not a surface protein. However, only a single enzyme was examined in these experiments and so it is possible that PGK may have cell wall localization in addition to Plr. It is interesting to note that the *B. stearothermophilus* GAPDH of strain 64/14k-53 in lane 2 of figure 3-7 is present in substantial quantity in the mutanolysin extract. This may either represent surface localization of this GAPDH or merely contamination of cytoplasmic contamination of the mutanolysin extracts.

Glycolytic enzymes have been reported to have surface localization in several organisms. Both GAPDH and triose phosphate isomerase have been reported to have cell wall localization in *Schistosoma mansoni* (Goudot-Crozel et al., 1989; Harn et al., 1988) GAPDH has also been reported on the surface of *Kluyveromyces marxianus* (Fernandes et al., 1992) and associated with the cell membrane of erythroid cells (Allen et al. 1987), alpha-enolase on the U937 monocytoid cells (Miles et al. 1991) and rat neuron membrane (Nakajima et al., 1994).

The leakage of cytoplasmic components into mutanolysin extracts has been previously demonstrated for *Streptococcus mutans* (Siegel et al., 1981). The percentage of bacterial lysis after 1 hr incubation with mutanolysin as measured by O.D. 600_{nm} was reduced from 88% to 23% lysis upon the addition

of 40% raffinose to buffer from that of untreated cells. When $MgCl_2$, a membrane stabilizer, was added to the buffer containing 40% raffinose only 7% lysis occurred. When the bacteria were grown in the presence of [^{3}H] thymidine and [^{14}C] leucine, the release of radiolabeled DNA and proteins followed a similar trend as seen for the lysis analysis by O.D. 600_{nm}.

In strain 64/14 there was no decrease in the relative quantity of PGK activity in the mutanolysin extract in buffer containing 30% raffinose compared to buffer alone suggesting that the PGK activity in mutanolysin extracts of group A streptococci with cytoplasmic proteins may be due to protoplast leakage and not cell lysis. Consistent with the hypothesis that cell leakage occurs upon cell wall removal, an anti-Plr polyclonal antibody reacted with intracellular protein in mutanolysin treated strain 64/14 using indirect immunofluorescence to visualize the primary antibody (data not shown).

Plr had been previously isolated from mutanolysin extracts of group A streptococci and was shown to bind plasmin by ligand blot assay. Analogous to the specificity of strain 64/14 for binding lys-plasmin or lys-plasminogen with minimal binding of glu-plasminogen, Plr preferentially binds plasmin. Based on these criteria it was hypothesized that Plr may have surface localization and serve as a plasmin receptor. Studies presented in the previous chapters have identified Plr as a streptococcal GAPDH enzyme. In vitro

characterization of recombinant Plr by site-specific mutagenesis of *plr* identified a C-terminal lysine residue that was necessary but not sufficient for wild-type levels of plasmin binding. Isogenic derivatives of group A strain 64/14 containing 3' end mutations in *plr* did not differ from wild-type strain 64/14 in the ability to capture plasmin. These results indicated that Plr may not be functioning as a plasmin receptor as originally hypothesized. Experiments were then performed to further examine plasmin binding components of strain 64/14 and to look for evidence supporting surface localization of Plr. Results indicate that the majority of plasmin binding components of strain 64/14 can be attributed to surface proteins. This binding may be at least partially mediated via C-terminal lysine residues of these proteins. However, surface localization of Plr has not been supported by the experiments presented here. Furthermore, finding a protein in mutanolysin extracts is not sufficient criteria for concluding that a protein has surface localization. Future studies examining Plr localization in group A streptococci using techniques such as immunofluorescence with the appropriate controls to monitor for non-specific binding activity, and electron microscopy with immunoferritin on intact cell wall preparations may more definitively address the surface exposure and/or cell wall-association of this GAPDH enzyme.

EPILOGUE

The group A streptococcus is a serious human pathogen which can cause significant morbidity and mortality. The primary treatment for group A streptococcal infections, as for most bacterial infections, is the utilization of antibiotics. However, because of the rapidity of bacterial spread in invasive infections and host mediated inflammatory responses in the toxic shock-like syndrome caused by group A streptococci, antibiotics are not always effective. Furthermore, with the widespread use of antibiotics, the threat of resistance becomes an ever increasing concern. In the case of group A streptococci, penicillin remains the only antibiotic where resistant strains have not yet been reported. Therefore it is important to study these bacteria to learn more about their virulence factors as well as their basic biology so that rational alternative treatments and preventative interventions can be developed.

The ability of group A streptococci to rapidly traverse tissue barriers in the course of an infection has been hypothesized to be mediated by the ability of these bacteria to interact with the host plasminogen system. Streptokinase, a plasminogen activator secreted by streptococci, efficiently converts plasminogen to the serine protease plasmin. Purified preparations of this

streptococcal protein are often used clinically to rapidly hydrolyze fibrin clots in the coronary arteries of patients experiencing myocardial infarction. The role of streptokinase in streptococcal pathogenesis has not yet been determined. Our laboratory has shown that group A streptococci, growing in the presence of human plasma, can activate the endogenous plasminogen and capture plasmin activity on the bacterial surface (Lottenberg et. al 1993). This surface bound proteolytic activity is no longer regulated by physiological plasmin inhibitors. To assess the importance of this system in the pathogenesis of group A streptococcal infections, plasmin receptor molecules need to be identified and characterized. The genes encoding these plasmin receptors and the gene encoding streptokinase can then be individually inactivated and studied in an animal model for bacterial invasion so that the contribution of each molecule to the invasive potential of the bacteria can be assessed.

A candidate plasmin receptor of group A streptococcal strain 64/14 was previously identified and the gene encoding this protein was cloned, sequenced, and expressed in *E. coli* (Lottenberg et al., 1992a) The gene exhibited homology to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). GAPDH is an enzyme of the pathway which metabolizes glucose to pyruvic acid. Glycolytic enzymes, including GAPDH, and the genes encoding them have been studied extensively in many organisms. Surprisingly

however, they have been characterized for very few pathogenic bacteria and not at all for group A streptococci. Perhaps because they are basic housekeeping enzymes they have been ignored, but knowledge regarding their regulation and essentiality in the growth of these pathogens should not be overlooked.

In the studies presented in this work, cytoplasmic GAPDH from group A streptococci has been isolated and compared to the putative plasmin receptor protein, Plr. Biochemical analyses of the primary amino acid sequences, as well as functional analyses indicated that Plr was identical to cytoplasmic GAPDH in strain 64/14. Furthermore, DNA hybridization analyses demonstrated that this protein is encoded by a single *plr* gene on the streptococcal chromosome. Attempts at inactivation *plr* in order to assess the role of Plr as a plasmin receptor were unsuccessful and indicated that *plr* may be an essential gene. Therefore, alternative strategies were required to examine the role of this potentially multi-functional GAPDH molecule. Studies were initiated to identify regions or residues that were involved in the ability of purified Plr to bind plasmin. Directed mutagenesis studies were performed on recombinant *plr*, and the expressed products were analyzed for plasmin binding ability. These studies demonstrated that a C-terminal lysine of Plr was necessary but not sufficient for wild-type levels of plasmin binding. It appeared that NH_2-terminal residues may also be required for the appropriate presentation of the

C-terminal lysine. Mutations of the C-terminal lysine retained GAPDH enzymatic activity. Because the GAPDH activity was potentially essential for viability of group A streptococci, strategies were developed to replace the wild-type *plr* gene with the genes encoding these plasmin binding deficient mutants. Isogenic strains containing these plr mutations expressed Plr which showed reduced plasmin binding ability. However, the ability of the bacteria to capture plasmin on the cell surface was unaffected in mutant strains compared to wild-type. These results indicated that Plr may not be a plasmin receptor or at least it was not the sole contributor to plasmin binding activity. Additional studies indicated that surface molecules other than Plr may modulate this activity.

Another laboratory has inactivated the gene encoding streptokinase and shown that the streptococci that do not express the plasminogen activator failed to capture plasmin on the cell surface when incubated in the presence of plasminogen (Malke et al., 1994).

Animal models to study the pathogenesis of streptococci have been limited because these bacteria appear to have evolved as species specific pathogens. This is exemplified by the selectivity of plasminogen activation by streptokinases of streptococci isolated form either humans or horses (McCoy et al., 1991). A mouse model for bacterial invasion has been developed (Raeder and Boyle, 1993). However, the ability of streptokinase produced by group A

streptococci to activate murine plasminogen efficiently remains as a potential problem. Furthermore, not all group A streptococcal strains are lethal in this mouse model. The continued development of transgenic animals may enhance the ability to define virulence factors with improved animal models.

Since I initiated my laboratory investigation, it has become clear that an alternative pathway leading to the capture of plasmin activity by streptococci exists and that there also exist at least two distinct surface structures mediating the direct binding of plasmin. It will be necessary to address streptokinases as well as surface plasmin(ogen) binding components to define the participation of the host plasminogen system in the tissue invasiveness exhibited by streptococci.

Alefounder, P. R., and R. N. Perham. 1989. Identification, molecular cloning and sequence analysis of a gene cluster encoding the Class II fructose 1,6-biphosphate aldolase, 3-phosphoglycerate kinase and a putative second glyceraldehyde 3-phosphate dehydrogenase of *Escherichia coli*. Mol. Microbiol. **3**:723-732.

Allen, R. W., K. A. Trach, and J. A. Hoch. 1987. Identification of the 37-kDa protein displaying a variable interaction with the erythroid cell membrane as glyceraldehyde 3-phosphate dehydrogenase. J. Biol. Chem. **262**:649-643.

Amann, E., B. Ochs, and K. J. Abel. 1988. Tightly regulated *tac* promoters useful for the expression of unfused and fused proteins in *Escherichia coli*. Gene **69**:301-315.

Aoki, N. 1995. Alpha-2-plasmin inhibitor. p. 545-549. *In* K. A. High and H. R. Roberts (ed), Molecular Basis of Thrombosis and Hemostasis. Marcel Dekker, Inc., New York City, New York.

Berge A., and U. Sjobring. 1993. PAM, a novel plasminogen-binding protein from *Streptococcus pyogenes*. J. Biol. Chem. **34**:25417-25424.

Bisno, A. L. 1991. Group A streptococcal infections and acute rheumatic fever. NEJM **325**:783-793.

Branlant, C., T. Oster, and G. Branlant. 1989. Molecular cloning of the glyceraldehyde-3-phosphate dehydrogenase genes of *Bacillus stearothermophilus* and *Escherichia coli*, and their expression in *Escherichia coli*. Gene **25**:1-7.

Branlant, G., G. Flesch, and C. Branlant. 1983. Nucletide sequence determination of the DNA region coding for *Bacillus stearothermophilus* glyceraldehyde-3-phosphate dehydrogenase and of the flanking DNA regions required for its expression in *Escherichia coli*. Gene **75**:145-155.

Broder, C. C., R. Lottenberg, and M. D. P. Boyle. 1989. Mapping of the human plasmin domain recognized by the unique plasmin receptor of group A streptococci. Infect. Immun. **57**:2597-2605.

Broder, C. C., R. Lottenberg, G. O. von Mering, K. H. Johnston, and M. D. P. Boyle. 1991. Isolation of a prokaryotic plasmin receptor: relationship to a plasminogen activator produced by the same micro-organism. J. Biol. Chem. **266**:4922-4928.

Broeseker, T. A., M. D. P. Boyle, and R. Lottenberg. 1988. Characterization of the interaction of human plasmin with its specific receptor on a group A streptococcus. Microbial Path. **5**:19-27.

Burge, S. M., J. M. Marshall, and S. A. Cederholm-Williams. 1992. Plasminogen binding sites in normal human skin. Brit. J. Derm. **126**:35-41.

Camacho, M., M. C. Fondaneche, and P. Burtin. 1989. Limited proteolysis of tumor cells increases their plasmin-binding ability. FEBS Letters **245**:21-24.

Castellino, F. J. 1979. A unique enzyme-protein substrate modified reaction: plasmin/streptokinase interaction Trends Biochem. Sci. **4**:1-5.

Castellino, F. J. 1995. Plasminogen. p. 495-515. *In* K. A. High and H. R. Roberts (ed), Molecular basis of Thrombosis and hemostasis. Marcel Dekker, Inc., New York City, New York.

Caparon, M. G., and J.R. Scott. 1991. Genetic manipulation of pathogenic streptococci. Methods Enzymol. **204**:556-586.

Chang, A. C. Y., and S. N. Cohen. 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles from the p15A cryptic miniplasmid. J. Bacteriol. **134**:1141-1156.

Christensen, U. 1985. C-terminal lysine residues of fibrinogen fragments essential for binding to plasminogen. FEBS Letters **182**:43-46.

Charrier-Ferrara, S., D. Caillol, and V. Goudot-Crozel. 1992. Complete sequence of the *Schistosoma mansoni* glyceraldehyde-3-phosphate dehydrogenase gene encoding a major surface antigen. Mol. and Biochem. Para. **56**:339-344.

Cleveland, D. W., S. G. Fischer, M. W. Kirschner, and U. K. Laemmli. 1977. Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. J. Biol. Chem. **252**:1102-1106.

Comer, M. J., D. B. Craven, M. J. Harvey, A. Atkinson, and P. D. G. Dean. 1975. Affinity chromatography on immobilized nucleotides. Eur. J. Biochem. **55**:201-209.

Conway, T., and L. O. Ingram. 1988. Phosphoglycerate kinase gene from *Zymomonas mobilus*: cloning, sequencing, and localization within the *gap* operon. J. Bacteriol. **170**:1926-1933.

DesJardin, L. E., M. D. P. Boyle, and R. Lottenberg. 1989. Group A streptococci bind human plasmin but not other structurally related proteins. Thromb. Res. **55**:187-193.

Drake, C. G., and B. L. Kotzin. 1992. Superantigens: biology, immunology, and potential role in disease. J. Clin. Immun. **12**:149-162.

Eikmanns, B. J. 1992. Identification, sequence analysis, and expression of a *Cornyebacterium glutamicum* gene cluster encoding the three glycolytic enzymes glyceraldehyde-3-phosphate dehydrogenase, 3-phosphoglycerate kinase, and triosephosphate. J. Bacteriol. **174**:6076-6086.

Fellay, R., J. Frey, and H. Kirsch. 1987. Interposon mutagenesis of soil and water bacteria: a family of DNA fragments designed for *in vitro* insertional mutagenesis of gram-negative bacteria. Gene **52**:147-154.

Ferdinand, W. 1964. The isolation and specific activity of rabbit muscle glyceraldehyde-3-phosphate dehydrogenase. Biochem. Sci. **92**:578.

Fernandes, P. A., J. N. Keen, J. B. C. Findlay, and P. Moradas-Ferreira. 1992. A protein homologous to glyceraldehyde-3-phosphate dehydrogenase is induced in the cell wall of a flocculent *Kluyveromyces marxianus*. Biochim. et Biophys. Acta. **1159**:67-73.

Fischetti, V. A. 1989. Streptococcal M protein: Molecular design and biological behavior. Microbiol. Rev. **2**:285-314.

Fuchs, H., R. Wallich, M. M. Simon, and M. D. Kramer. 1994. The outer surface protein A of the spirochete *Borrelia burdorferi* is a plasmin(ogen) receptor. Proc. Natl. Acad Sci, USA. **91**:12594-12598.

Galan, J.E., J. F. Timoney, and R. Curtiss III. 1988. Expression and localization of the *streptococcus equi* M-protein in *Escherichia coli* and *Salmonella typhimurium*, p. 34-40. In D. G. Powell (ed.), Proceeding of the Fifth International Conference of Equine Infectious Diseases. University Press of Kentucky, Lexington Ky.

Goudot-Crozel, V., D. Caillol, M. Djabali, and A. L. Dessein. 1989. The major parasite surface antigen associated with human resistance to schistosomiasis is a 37-kD glyceraldehyde-3p-dehydrogenase. J. Exp. Med. **170**:2065-2080.

Hajjar, K. A. 1991. The endothelial cell tissue-plasminogen activator receptor: specific interaction with plasminogen. J. Biol. Chem. **266**:21962-21970.

Hajjar, K. A. 1993. Homocysteine modulation of tissue-plasminogen activator binding to its endothelial cell membrane receptor. J. Clin. Invest. **91**:2873-2879.

Hajjar, K. A. 1994. An endothelial cell receptor for tissue-plasminogen activator. I. Identity with annexin II. J. Biol. Chem. **269**:21191-21197.

Hannaert, V., M. Blaauv, L. Kohl., Allert, S., Opperdoes, F. R., and P. A. M. Michels. 1992. Molecular analysis of the cytosolic and glycosomal glyceraldehyde-3-phosphate dehydrogenase in *Leishmania Mexicana*. Mol. and Biochem. Para. **55**:115-126.

Hanski, E, and M. Caparon. 1992. Protein F, a fibronectin-binding protein, is an adhesin of the group A streptococcus *Streptococcus pyogenes*. Proc. Natl. Acad. Sci. USA **89**:6172-6176.

Harn, D. A., A. Gebremichael, L. Oligino, N. Brown, and E. D. Huguenel. 1988. A 28-kDa antigen from *S. mansoni* shares sequence homology with a mammalian glycolytic enzyme. FASEB J. Abstr. A1256.

Harris, J. I., J. D. Hocking, M. J. Runswick, K. Suzuki, and J. E. Walker. 1980. D-glyceraldehyde-3-phosphate dehydrogenase, the purification and characterization of the enzyme from the thermophiles *Bacillus stearothermophilus* and *Thermus aquaticus*. Eur. J. Biochem. **108**:535-547.

Harris, J. I., and M. Waters. 1976. Glyceraldehyde-3-phosphate dehydrogenase. Enzymes **13**:1-49.

Hauser, A. R., D. L. Stevens, E. L. Kaplan, and P. M. Schlievert. 1991. Molecular analysis of pyrogenic exotoxins from *Streptococcus pyogenes* isolates associated with toxic shock-like syndrome. J. Clin. Micro. **29**:1562-1567.

Henikoff, S. 1987. Unidirectional digestion with exonuclease III in DNA sequence analysis. Methods Enzymol. **155**:156-166.

Holland, J. P. 1983. Homologous nucleotide sequence at the 5' termini of messenger RNAs synthesized from the yeast enolase and glyceraldehyde-3-phosphate dehydrogenase gene families. J. Biol. Chem. **258**:5291-5299.

Horecker, B.L., and A. Kornberg. 1948. The extinction coefficient of the reduced band of pyridine nucleotides. J. Biol. Chem. **175**:385-390.

Hortin, G. L., B. L. Trimpe, and K. F. Folk. 1989. Plasmin's peptide-binding specificity: characterization of ligand sites in alpha-2-antiplasmin. Throm. Res. **54**:621-632.

Johnson, R. H. 1975. Characterization of group A streptococcal R-28 antigen purified by hydroxyapatite column chromotography. Infect. Immun. **12**:901-909.

Kapur, V., M. W. Majesky, L. L. Li, R. A. Black, and J. M. Musser. 1993. Cleavage of interleukin 1B (IL-1B) precursor to produce active IL-1 by a conserved extracellular cysteine protease from *Streptococcus pyogenes*. Proc. Natl. Acad. Sci. USA **90**:7676-7680.

Kuusela, P., and O. Saksela. 1990. Binding and activation of plasminogen at the surface of *Staphylococcus aureus*. Eur. J Biochem. **193**:759-765.

Kuusela, P., M. Ullberg, G. Kronvall, T. Tervo, A. Tarkkanen, and O. Saksela. 1992a. Surface-associated activation of plasminogen on Gram positive bacteria. Acta Ophthal. **70**:42-46.

Kuusela, P., M. Ullberg, O. Saksela, and G. Kronvall. 1992b. Tissue-type plasminogen activator-mediated activation of plasminogen on the surface of group A, C, and G streptococci. Infect. Immun. **60**:196-201.

Lahteenmaki, K. , B. Westerlund, P. Kuusela, and T. K. Korhonen. 1993. Immobilization of plasminogen on *Escherichia coli* flagella. FEMS Microbiol. Lett. **106**:309-314.

Lammeli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**:680-685.

Lee, P.K., and P. M. Schlievert. 1989. Quantification and toxicity of group A streptococcal pyrogenic exotoxins in an animal model of toxic shock syndrome. J. Clin. Microbiol. **27**:18990-1892.

Leong, J. M., R. S. Fournier, and R. R. Isberg. 1991. Identification of the integrin binding domain of the *Yersinia pseudotuberculosis* invasin protein. EMBO J. **9**:1979-1989.

Lopez-Alemany, R., P. Correc, L. Camoin, and P. Burtin. 1994. Purification of the plasmin receptor from human carcinoma cells and comparison to alpha-enolase. Thromb. Res. **75**:371-381.

Lottenberg, R., C. C. Broder, and M. D. P. Boyle. 1987. Identification of a specific receptor for plasmin on a group A streptococcus. Infect. Immun. **55**:1914-1928.

Lottenberg, R., C. C. Broder, M. D. P. Boyle, S. J. Kain, B. L. Schroeder, and R. Curtiss III. 1992. Cloning, sequence analysis, and expression in *Escherichia coli* of a streptococcal plasmin receptor. J. Bacteriol. **174**:5204-5210.

Lottenberg, R., L. E. DesJardin, H. Wang, and M. D. P. Boyle. 1992. Streptokinase-producing streptococci grown in human plasma acquire unregulated cell-associated plasmin activity. J. Infect. Dis. **166**:436-440.

Lottenberg, R., D. Minning-Wenz, and M. D. P. Boyle. 1994. Capturing host plasmin(ogen): a common mechanism for invasive pathogens? Trends in Micro. **2**:20-24.

Lucas, M. A., L. J. Fretto, and P. A. Mckee. 1983. The binding of human plasminogen to fibrin and fibrinogen. J. Biol. Chem. **258**:4249-4256.

Maitra, P. K., and Z. Lobo. 1971. A kinetic study of glycolytic enzyme synthesis in yeast. J. Biol. Chem. **246**:475-488.

Malke, H., U. Mechold, K. Gase, and D. Gerlach. 1994. Inactivation of the streptokinase gene prevents *Streptococcus equisimilis* H46A from acquiring cell-associated plasmin activity in the presence of plasminogen. FEMS Microbiol. Letters. **116**:107-112.

Maniatis, T., E. F. Fritsch, and J. Sambrook. 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

McCoy, H. E., C. C. Broder, and R. Lottenberg. 1991. Streptokinases produced by pathogenic group C streptococci demonstrate species-specific plasminogen activation. J. Infect. Dis. **164**:515-521.

Michels, P. A. M., M. Marchando, L Kohl, S. Allert, R. K. Wierenga, and F. R. Opperdoes. 1991. The cytosolic and glycosomal isoenzymes of glyceraldehyde-3-phosphate dehydrogenase in *Trypanosoma brucei* have a distant evolutionary relationship. Eur. J. Biochem. **198**:421-428.

Michels, P. A. M., A. Poliszczak, K. A. Osinga, O. Misset, J. Van Beemen, R. K. Wierenga, P. Borst, and F. R. Opperdoes. 1986. Two tandemly linked identical genes code for the glycosomal glyceraldehyde-3-phosphate dehydrogenase in *Trypanosoma brucei*. EMBO J. **5**:1049-1056.

Miles, L. A., C. M. Dahlberg, J. Plescia, J. Felez, K. Kato, and E. F. Plow. 1991. Role of cell-surface lysines in plasminogen binding to cells: identification of alpha-enolase as a candidate plasminogen receptor. Biochemistry **30**:1682-1691.

Miles, L. A., M. H. Ginsberg, J. G. White, and E. F. Plow. 1986. Plasminogen interacts with human platelets through two distinct mechanisms. J. Clin. Invest. **77**:2001-2009.

Miles, L. A., and E. F. Plow. 1988. Plasminogen receptors: ubiquitous sites for cellular recognition of fibrinolysis. Fibrinolysis **2**:61-71.

Nakajima, K., M. Hamanoue, N. Takemoto, T. Hattori, K. Kato, and S. Kohsaka. 1994. Plasminogen binds specifically to alpha-enolase on rat neuronal plasma membrane. J. Neurochem. **63**:2048-2057.

Nasar, B. A., A. Wistedt, U. Ringdahl, and U. Sjobring. 1994. Streptokinase activates plasminogen bound to human group C and G streptococci through M-like proteins. Eur. J. Biochem. **222**:267-276.

Nelson, K., T. S. Whittam, and R. K. Selander. 1991. Nucleotide polymorphism and evolution in the glyceraldehyde-3-phosphate dehydrogenase gene (gapA) in natural populations of *Samonella* and *Escherichia coli*. Proc. Natl. Acad. Sci. USA **88**:6667-6671.

Nieuwenhuizen, W., A. Vermond, M. Voshkuilen, D. W. Traas, and J. H. Vereijen. 1983. Identification of a site in fibrin(ogen) which is involved in the acceleration of plasminogen activiation by tissue-type plasminogen activator. Biochim. Biophys. Acta. **748**:86-92.

Okada, N., R. Geist, and M. G. Caparon. 1993. Positive transcriptional control of *mry* regulates virulence in the group A streptococcus. Mol. Microbiol. **7**:893-903.

Okada, N., A. P. Pentland, P. Falk, and M. G. Caparon. 1994. M Protein and Protein F act as important determinants of cell-specific tropism of Streptococcus pyogenes in skin tissue. J. Clin. Invest. **94**:965-977.

Pancholi, V., and V. A. Fishchetti. 1988. Isolation and characterization of the cell-associated region of group A streptococci M6 protein. J. Bacteriol. **170**:2618-2624.

Pancholi, V., and V. A. Fishchetti. 1992. A major surface protein on group A streptococci is a glyceraldehyde-3-phosphate dehydrogenase with multiple binding activity. J. Exp. Med. **176**:415-426.

Pancholi, V.,and V. A. Fischetti 1993. Glyceraldehyde-3-phosphate dehydrogenase on the surface of group A streptococci is also an ADP-ribosylating enzyme. Proc. Natl. Acad. Sci. USA **90**:8154-8158.

Pannell, R., J. Black, and V. Gurewich. 1988. Complementary modes of action of tissue-type plasminogen activator and pro-urokinase by which their synergistic effect on clot lysis may be explained. J. Clin. Invest. **81**:853-859.

Parkkinen, J., and T. K. Korhonen. 1989. Binding of plasminogen to *Escherichia coli* adhesion proteins. FEBS **250**: 437-440.

Poirier, T. P. 1989. Fibrinogen binding and resistance to phagocytosis of *Streptococcus sanquis* expressing cloned M protein of *Streptococcus pyogenes*. Infect. and Immun. **57**:29-35.

Raeder, R and M. D. P. Boyle. 1993. Association between expression of immunoglobulin G-binding proteins by group A streptococci and virulence in a mouse skin infection model. Infect.Immun. **61**:1378-1384.

Reis, K., E. M. Ayoub, and M. D. P. Boyle. 1983. Detection of receptors for Fc region of IgG on streptococci. J. Immunol. Methods **59**:83.

Reis, K., M. Yarnall, E. M. Ayoub, and M. D. P. Boyle. 1984. Effect of mouse passage on Fc receptor expression by group A streptococci. Scand J. Immunol. **20**:433-439.

Sakai, K., K. Hasumi, and A. Endo. 1990. Two glyceraldehyde-3-phosphate dehydrogenase isozymes from the koningic acid (heptelidic acid) producer *Trichoderma koningii*. Eur. J. Biochem. **193**:195-202.

Sasaki, T., T. Morita, and S. Iwanaga. 1986. Identification of the plasminogen-binding site of human alpha-2-plasminogen inhibitor. Eur. J. Biochem. **193**:195-202.

Schlapfer, B. S., and H. Zuber. 1992. Cloning and sequencing of the genes encoding glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase and triose isomerase (*gap* operon) from mesophilic *Bacillus megaterium*: comparison with corresponding sequences from thermophilic *Bacillus stearothermophilus*. Gene **122**:53-62.

Schwinde, J. W., N. Thum-Schmitz, B. J. Eikmanns, and H. Sahm. 1993. Transcriptional analysis of the *gap-pgk-tpi-ppc* gene cluster of *Cornyebacterium glutamicum*. J. Bacteriol. **175**:3905-3908.

Segel, I. H. 1976. Biochemical Calculations, 2nd Ed. p. 416. John Wiley and Sons, New York City, New York.

Siegel, J. L., S. F. Hurst, E. S. Liberman, S. E. Coleman, and A. S. Bleiweis. 1981. Mutanolysin-induced spheroplasts of *Streptococcus mutans* are true protoplasts. Infect. Immun. **31**:808-815.

Simon, D., and J.J. Ferretti. 1991. Electrotransformation of *Streptococcus pyogenes* with plasmid and linear DNA. FEMS Microbiology Letters. **82**:219-224.

Simpson, D., and E. H. Beachey. 1983. Adherence of group A streptococci to fibronectin on oral epithelial cells. Infect. Immun. **39**:275-279.

Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503-517.

Stevens, D. L. 1992. Invasive group A infections. Clin. Infect. Dis. **14**:2-13.

Sugiyama, N., T. Sasaki, M. Iwamoto, and Y. Abiko. 1988. Binding site of alpha-2-plasmin inhibitor to plasminogen. Biochimica. et. Biophysica Acta. **952**:1-7.

Ullberg, M., G. Kronvall, I. Karlson, and B. Wiman. 1990. Receptors for human plasminogen on gram negative bacteria. Infect. and Immun. **58**:21-25.

Ullberg, M., G. Kronvall, and B. Wiman. 1989. New receptors for human plasminogen on gram positive cocci. APMIS **97**:996-1002.

Ullberg, M., P. Kuusela, B-E. Kristeansen, and G. Kronvall. 1992. Binding of plasminogen to *Neisseria meningitidis* and *Neisseria gonorrhoeae* and formation of surface-associated plasmin. J. Infect. Dis. **166**:1329-34.

Van DeRijn, I, and R. E. Kessler. 1980. Growth characteristics of group A streptococci in a new chemically defined medium. Infect. Immun. **27**:444-448.

Vassalli, J. D., D. Baccino, and D. Belin. 1985. A cellular binding site for the Mr 55,000 form of the human plasminogen activator, urokinase. J. Cell. Biol. **100**:86-92.

Vassalli, J. D., A. P. Sappino, and D. Belin. 1991. The plasminogen activator/plasmin system. J. Clin. Invest. **88**:1067-1072.

Violand, B. N., J. M. Sodetz, and F. J. Castellino. 1975. The effect of epsilon aminocaproic acid on the gross conformation of plasminogen and plasmin. Arch. Biochem. Biophys. **170**:300-305.

Visai, L., S. Bozzini, G. Raucci, A. Toniolo, P. Speziale. 1995. Isolation and characterization of a novel collagen-binding protein from *Streptococcus pyogenes* strain 64 14. J. Biol. Chem. **270**:347-353.

Wang, H, R. Lottenberg, and M. D. P. Boyle. 1994. Analysis of plasmin(ogen) acquisition by clinical isolates of group A streptococci incubated in human plasma. J. Infect. Dis. **169**:143-149.

Wessels, M. R., A. E. Moses, J. B. Goldberg, and T. J. DiCesare. 1991. Hyaluronic acid capsule is a virulence factor for mucoid group A streptococci. Proc. Natl. Acad. Sci. USA **88**:8317-8321.

Wiman, B., H. R. Lijnen, and D. Collen. 1979. On the specific interaction between the lysine-binding sites in plasmin and complementary sites in alpha-2-antiplasmin and in fibrinogen. Biochim. Biophys. Acta. **579**:142-154.

Wolf, B. B., C. A. Gibson, V. K. Kapurt, I. M. Hussaini, J. M. Musser, and S. L. Gonias. 1994. Proteolytically active streptococcal pyrogenic exotoxin B cleaves monocytic cell urokinase receptor and releases an active fragment of the receptor from the cell surface. J. Biol. Chem. **269**:30682-30687.

Yarnall, M. and M. D. P. Boyle. 1986. Isolation and partial characterization of a type II Fc receptor from a group A streptococcus. Mol. Cell. Biochem. **70**:57-66.

BIOGRAPHICAL SKETCH

Scott Budd Winram was born in Philadelphia, Pennsylvania, on December 8, 1964. He is the second of two children. He lived in Wilmington, Delaware, until attending college at the University of Delaware in Newark, Delaware. He graduated in 1987 with a Bachelor of Arts degree in biology. He worked as a laboratory technician at the Stroud Water Research Institute in Avondale, Pennsylvania, and then at the University of Delaware before beginning his graduate studies at the University of Florida in 1990. He has accepted a postdoctoral fellowship position in the laboratory of Dr. Craig Rubens studying the pathogenesis of group B streptococci.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Richard Lottenberg, Chair
Molecular Genetics and
Microbiology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Paul A. Gulig
Associate Professor of
Molecular Genetics and
Microbiology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Henry V. Baker
Associate Professor of
Molecular Genetics and
Microbiology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Michael S. Kilberg
Professor of Biochemistry
and Molecular Biology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Lonnie O. Ingram
Professor of Microbiology
and Cell Science

This dissertation was submitted to the Graduate Faculty of the College of Medicine and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy

August, 1995

Dean, College of Medicine

Dean, Graduate School